Measurement of LDL Receptor Activity in HepG2 Cells Grown on Microcarrier Beads

Thesis

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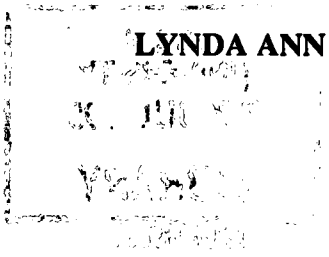
MEASUREMENT OF LDL RECEPTOR ACTIVITY IN HepG2 CELLS GROWN ON MICROCARRIER BEADS

LYNDA ANN DOVEY



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PREFACE

The experimental work reported in this dissertation was carried out in the Department of Pathology, University of Cambridge, between April 1992 and April 1994. This dissertation is the result of my own work, none of which has been submitted for a degree, diploma or other qualification here or at any other University and includes nothing which is the outcome of collaboration.

CONTENTS

ACKNOWLEDGEMENTS

I am grateful to Rhône-Poulenc Rorer for allowing me to use work carried out, whilst in their employment at Cambridge University, in this thesis.

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Last, but not least I would like to thank my husband Jim and children Cheryl and Hannah for their moral support and encouragement throughout this period of study.

MEASUREMENT OF LDL RECEPTOR ACTIVITY IN HepG2 CELLS GROWN ON MICROCARRIER BEADS

(Abstract)

Atherosclerosis- related conditions, coronary heart disease (CHD), stroke and peripheral arterial occlusions are the most important cause of death and invalidism in the Western world. A high plasma cholesterol level is a major risk factor for atherosclerosis. One of the main determinants of the plasma cholesterol level is the efficiency with which low density lipoprotein (LDL) is removed from the plasma via the hepatic LDL receptor. It is therefore important to study the factors controlling the expression of the receptor.

In the past, human hepatocytes have been grown in vitro as a monolayer culture in tissue culture dishes. This system permits the identification of substances that regulate the receptor activity but is limited as a research tool because substances secreted by the cells, such as bile acids and LDL itself, may cause feedback regulation of the pathway.

This thesis describes an alternative approach, in which HepG2 cells are grown on microcarrier beads which can then be packed into a column so that substances secreted by the cells can be removed by perfusion.

The study consists of four main sections:- i) Optimising conditions for establishing HepG2 cells on Cytodex microcarrier beads (discussed in Chapter 3); ii) Development of a column system for perfusion, either constant fresh medium (single pass) or by medium being constantly recycled (reperfusion) (discussed in Chapter 4); iii) Investigation of ligands which were available for the measurement of the LDL receptor to find the most suitable for use in this system (discussed in Chapter 5); iv) Perfusion column system,where some of the effects of single pass perfusion and re-perfusion were observed (discussed in Chapter 6).

HepG2 cells grown on Cytodex 2 microcarrier beads and perfused over various time periods were found to express lower LDL receptor activity when fed by re-perfusion compared to single pass perfusion. This did not appear to be due to depletion of essential nutrients, but to substances secreted by cells.

25 hydroxycholesterol used in this system was shown to regulate the LDL receptor. However, the extent of regulation was found to alter depending on whether the cells were fed by single pass perfusion or re-perfusion.

ABBREVIATIONS

α	Alpha.
AMP	Adenosine monophosphate.
apo	Apolipoprotein A.
apo E	Apolipoprotein E.
apo B	Apolipoprotein B-100.
ß	Beta.
BLPDS	Bovine Lipoprotein Deficient Serum.
BSA	Bovine Serum Albumin.
C	Centigrade.
C7	Monoclonal antibody (directed against the LDL receptor).
CHD	Coronary Heart Disease.
Ci, mCi, μCi	Curies, curies $x10^{-3}$, curies $x10^{-6}$.
cm^2	Square centimetres.
cpm	Count per minute.
DNA	Deoxyribonucleic acid.
DMSO	Dimethylsulphoxide.
FCS	Foetal Calf Serum.
FH	Familial Hypercholesterolemia.
g, mg, μg, ng.	Grammes, grammes x 10^{-3}, grammes x 10^{-6}, grammes x 10^{-9}.
x g	x centrifugal force.
HDL	High Density Lipoprotein.
HepG2	Human hepatic cell line.
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulphonicacid.
HMG CoA	3-hydroxy-3-methylgutaryl coenzyme A.

HSA	Human Serum Albumin.
^{125}I	Iodine 125.
IDL	Intermediate Density Lipoprotein.
I.U.	International Units.
l, ml, µl.	Litres, litres $x10^{-3}$, litres $x10^{-6}$.
kd	Kilodaltons.
LDL	Low Density Lipoprotein.
LPDS	Lipoprotein Deficient Serum.
M, mM. µM, nM, pM, fM.	Molar, molar $x10^{-3}$, molar $x10^{-6}$, molar $x10^{-9}$, molar $x10^{-12}$, molar $x10^{-15}$.
Mr	Molecular weight.
mRNA	Messenger ribonucleic acid.
Myb^{2-3}	Monoclonal antibody (not directed against the LDL receptor).
P	Probability studies.
PBS	Phosphate buffered saline.
rpm	revolutions per minute.
rmLDL	reductively methylated LDL.
RPMI $_{1640}$	10mM Hepes-bicarbonate buffered RPMI $_{1640}$ containing 100 i.u./ml Penicillin G and 100µg/ml Streptomycin.
R10 + Glu	RPMI $_{1640}$ as above with the addition of 10% FCS and L-Glutamine at 6mM.
SD	Standard deviation.
s.e.m.	Standard error of the mean.
S.E.M.	Scanning electron microscopy.
SDS	Sodium dodecyl sulphate.
V	Volts.
VLDL	Very Low Density Lipoprotein.

WHHL	Watanabe Heritable Hyperlipidemic Rabbit.
w/v	Weight / volume.

Chapter I

INTRODUCTION

1.1.1. Why Atherosclerosis is important.

Atherosclerosis-related conditions, coronary heart disease (CHD), stroke and peripheral arterial occlusions are the most important cause of death and invalidism in the Western world. In 1987 coronary heart disease accounted for 31% of deaths in the 45-54 year age group in England and Wales (HMSO 1987). It is a disease which predominately affects males although in females after menopause the incidence progressively approaches that in men. [DeBakey 1978]. Atherosclerosis rarely occurs in non-human primates and other animals.

1.1.2. Definition of Atherosclerosis [DeBakey 1978].

The World Health Organisation (1958) defines atherosclerosis as "A variable combination of changes in the intima of arteries consisting of the focal accumulation of lipid and complex carbohydrates with blood and its constituents, accompanied by fibrous tissue formation, calcification and associated changes in the media".

1.1.3. Histology and Pathophysiology.

In man atherosclerotic lesions primarily affect large and medium size arteries, particularly the coronary arteries and the abdominal aorta [Campbell and Chamley-Campbell 1981]. Eventually the focal lesions characteristic of this disease may cause vascular stenosis or may rupture resulting in rapid thrombotic reaction and occlusion. Such occlusion if it occurs in the coronary arteries, may produce the clinical condition of coronary heart disease, angina pectoris, myocardial infarction and acute cardiac death. Occlusions of the carotid arteries may give rise to cerebrovascular insufficiency, and stroke.

The progress of the disease involves accumulation of lipid and the formation of lipid-laden macrophages, migration of smooth muscle cells from the media with their subsequent proliferation in the intima and accompanying synthesis of fibrous tissue. Platelet aggregation may occur in an area of endothelial dysfunction.

Lesions (or plaques) may be classified into 5 various categories: [Bowyer, D. E. and Mitchinson, M.J. 1989]

a. *Grey gelatinous elevations;* local lesions with intimal oedema containing plasma constituents e.g. fibrinogen, glycosaminoglycans and some collagen. It is thought that these are early lesions in which lipid has not yet accumulated [Geer and Haust 1972, Gresham 1987].

b. *Fatty dots or streaks.* These appear as small well-demarcated yellow dots or streaks, which are flat or slightly raised and contain large amounts of lipid both intra- and extracellularly [Smith et al., 1967]. Both macrophages and some smooth muscle cells are found in these lesions, along with lipid engorged "foam cells" [McGill, 1968]. Many cells are engorged with lipid and are termed foam cells [McGill, 1968]. These are predominantly monocyte derived macrophages [Aqel et al., 1985]. Lipid-containing smooth muscle cells may also be present but they rarely have such a foamy appearance and are probably best referred to as lipid-rich or lipid-containing smooth muscle cells.

Gelatinous lesions, fatty dots and streaks are usually seen in adolescents and young adults.

c. *Fibrous plaques.* These are raised lesions usually covered with a dense fibrous cap of smooth muscle and connective tissue. Underneath the cap the lesions are composed mainly of smooth muscle cells with macrophages and some other leukocytes, identified as T-lymphocytes [Hansson et al., 1988; Jonasson et al., 1986]. These cells are surrounded by a connective tissue matrix and variable amounts of lipid droplets which may be intra- or

extracellular [Ross et al., 1984]. Necrotic debris, cholesterol crystals and calcification may occur beneath the cell rich region.

d. *Atheromatous lesions.* These have a central core of lipid and necrotic debris covered by a fibrous cap of smooth muscle cells.

e. *Advanced lesions.* These are atheromatous lesions complicated by ulcerations, thrombosis and/or calcification [Campbell and Chamley-Campbell, 1981].

The earliest lesions to be formed are the grey gelatinous elevations and the fatty streaks. It is unclear whether these develop into the more complicated lesions, that is fibrous plaques and atheromatous lesions observed later in life [Campbell and Chamley-Campbell, 1981]. Although the fibrous plaques and atheromatous lesions are usually grouped into two different categories there is in fact a continuum between these two classes.

1.1.4. Development of Atherosclerosis.

The development of atherosclerosis is generally characterised in the following way; see Fig. 1.1. [Bowyer, D.E. Lectures 1992.; Bowyer, D.E. and Mitchinson, M. J. 1989]

a. Macrophage infiltration of the arterial wall.

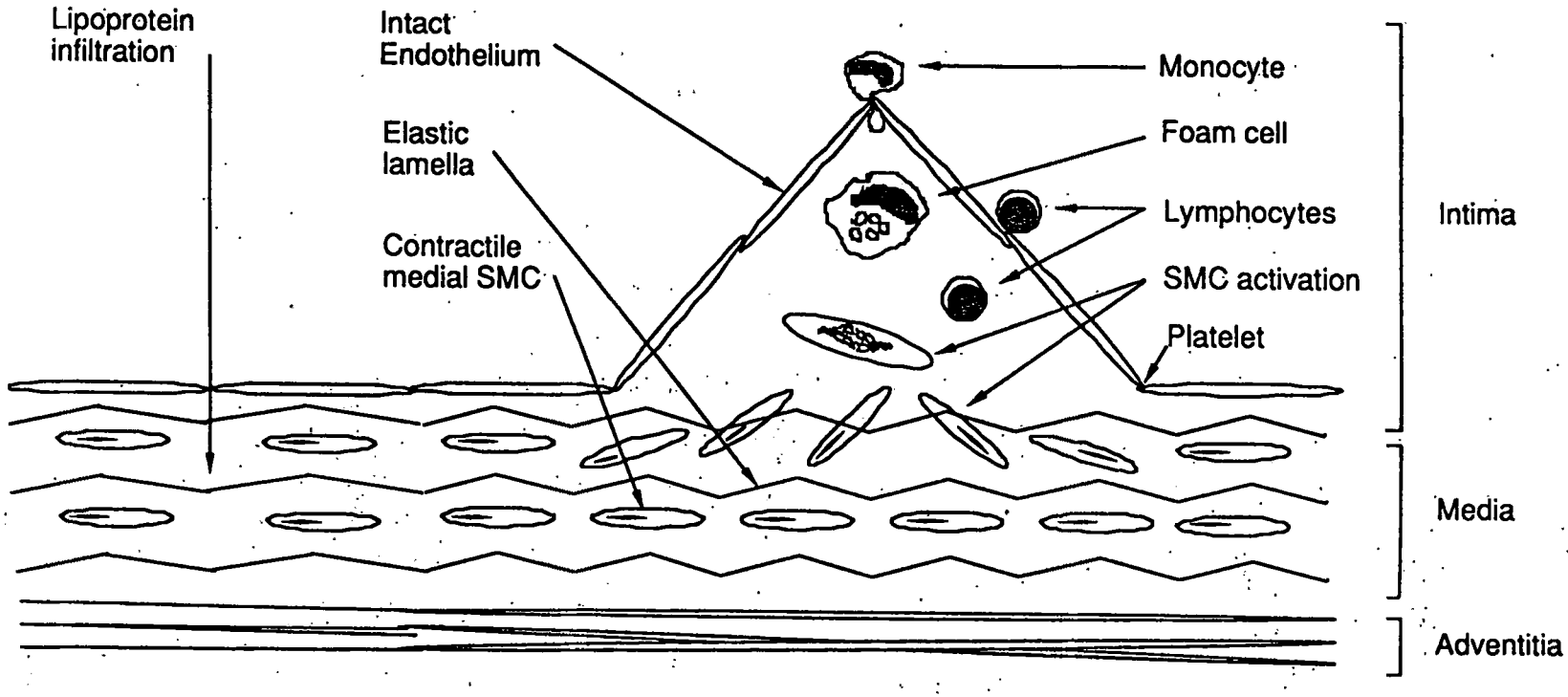
b. Appearance of "foam cells".

c. Migration of smooth muscle cells from the media into the intima and subsequent proliferation.

d. Synthesis by the smooth muscle cells of connective tissue components.

e. Deposition of extra- and intra-cellular lipid [Ross, 1984].

f. Infiltration of lipoproteins.



Processes in atherogenesis

[D.E. Bowyer: Lectures 1992]

Figure 1.1

1.1.5. Risk Factors.

Epidemiological studies (Neaton et al., 1984; Solberg and Strong, 1983) have all provided evidence for dividing risk factors into two categories i.e. primary and secondary factors.

a. Primary factors: These are considered to be capable, individually, of producing atherosclerotic vascular disease and its clinical complications:-

   Hyperlipoproteinemia and dyslipoproteinemia

   Smoking

   Hypertension.

b. Secondary risk factors: These factors are considered not to be capable individually, but only in conjunction with one or more other factors by producing complications of atherosclerotic vascular disease. Although over 200 different risk factors have shown statistically to correlate with atherosclerotic related diseases [Hopkins and Williams, 1981] the most important are thought to be:-

   Diabetes Mellitus

   Obesity

   Lack of physical exercise

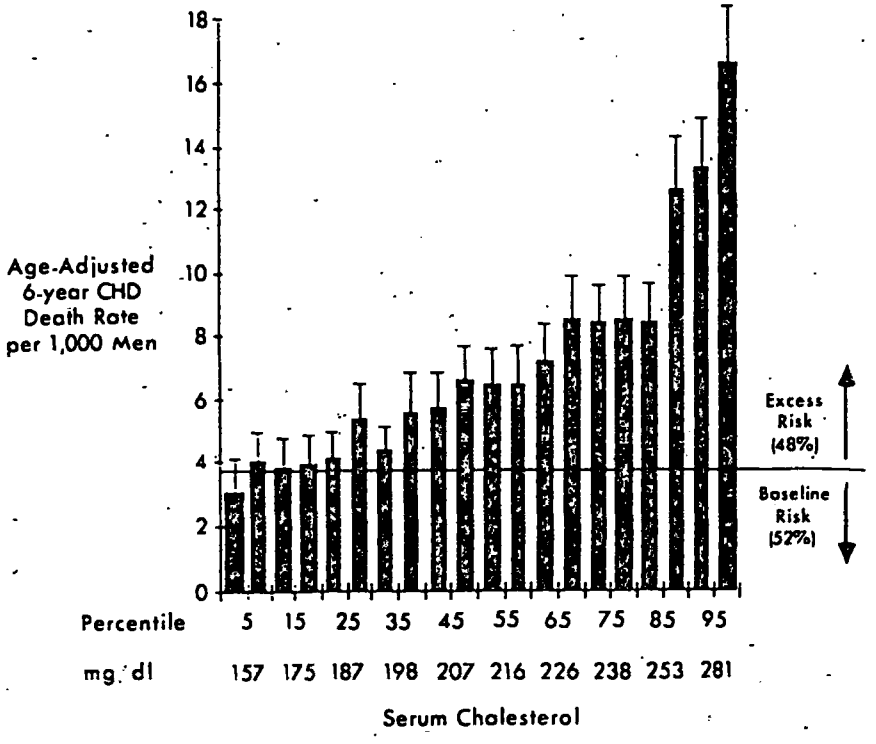
   Hyperuricemia.

1.1.6. Cholesterol and Atherosclerosis.

In the review by Bowyer [Bowyer 1967] cholesterol was emphasised as a major component of the atherosclerotic lesions. Early clinical observations of patients with coronary heart disease showed higher serum cholesterol concentrations than normal individuals [Lerman and White, 1946]. These studies generated such interest that later large human epidemiological studies were done to further prove the positive correlation of the high level of plasma cholesterol and the risk of atherosclerosis. One of these trials "the seven countries trial" showed that the mortality from CHD in different countries corresponded to the average cholesterol level in blood of its population. The CHD in

Japan, where only 7% of men had a serum level above 250mg/dl, is approximately 10% of the level observed in Finland where 56% of men had serum cholesterol levels over 250mg/dl [Keys 1970]. Some may say this could be due to the genetical difference between the two races of people. However, strong evidence for a dietary rather than purely genetic basis for the disease was provided by the observation that Japanese people living in Hawaii and California had a higher risk of having CHD than those living in Japan. Similarly, on an individual basis, Martin [Martin et al., 1986] analysed the 6 year mortality data for the 361,662 men screened as part of the "Multiple Risk Factor Intervention Trial" (MRFIT). This showed that with increasing plasma cholesterol levels there was a log-linear relationship with the risk from atherosclerosis. When the group was split into percentiles, it was found that above the 20th percentile, i.e. > 181 mg/dl, the risk of CHD increased as the serum level rose. If the baseline risk was taken as those below the 20th percentile, then 48% of all CHD deaths were associated with raised serum cholesterol levels of which 54% occurred between the 20th and the 85th percentile. The 15% of people with cholesterol levels above the 85th percentile i.e. > 253mg/dl showed a marked increase in risk of CHD. (See Figure 1.2).

Studies have been done to prove that lowering plasma cholesterol levels lowers atherosclerotic related illnesses. In animals in which atherosclerosis has been induced by a high cholesterol diet, the atherosclerotic lesions have been shown to regress if the animals are subsequently fed a low cholesterol diet [Small, 1988]. Furthermore, Blankenhorn and co-workers have demonstrated regression of atherosclerosis as indicated by percentile improvement of the overall coronary status in 16.2% of patients in which their plasma cholesterol level was lowered by colestipol and niacin treatment for three years compared to 2.4% in the placebo group [Blackenhorn et al., 1987]. Also, a decrease in the mortality from CHD has been observed in several studies when the plasma cholesterol level has been lowered through dietary and drug treatment. For example, the Helsinki Heart Studies have demonstrated a decrease in the mortality from CHD from 41/1000 to 27/1000 in control and treatment groups [Frick et al., 1987].



Correlation between serum cholesterol and the mortality rate from coronary heart disease (CHD)

Data from the multiple Risk Factor Intervention Trial.

[Martin et al., 1986]

Figure 1.2

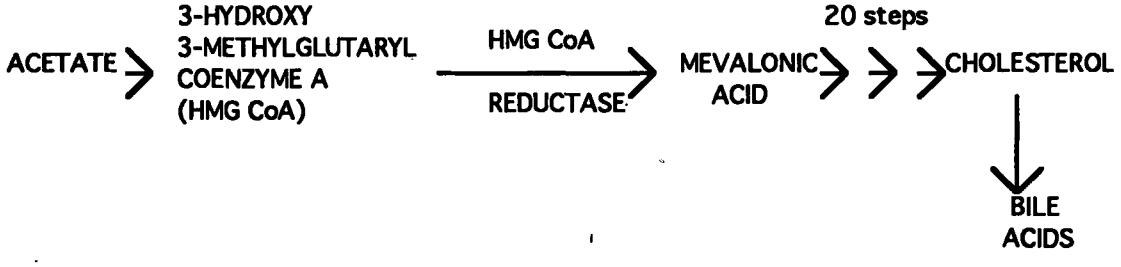
1.1.7. Cholesterol Absorption.

Cholesterol is very insoluble in aqueous systems. Within the intestine cholesterol is solubilized into mixed micelles containing fatty acids and monoglycerides (derived from hydrolysis of dietary triglycerides), lecithin, lysolecithin and bile salts. These lipids keep cholesterol in solution and bring it into proximity with the mucosal cells. Cholesterol then traverses a thin unstirred water layer by monomolecular diffusion to enter the outer membrane of the intestinal mucosal cells. All polar lipids participate in the solubilization of cholesterol, but only bile acids are essential. When the polar lipids are absorbed, cholesterol comes out of solution and cannot be reabsorbed.

1.1.8. Hepatic synthesis and degradation of cholesterol.

The liver is a major site of cholesterol synthesis in the body, although cholesterol is produced in many other organs and tissues.

In a normal Western diet the dietary cholesterol is not sufficient for supplying the enterohepatic system with bile acids and for VLDL synthesis simultaneously. Therefore approximately 75% of cholesterol needed for maintaining both pathways is derived from newly synthesised cholesterol by the liver, ultimately from acetate.



In this process HMGCoA reductase (which is a microsomal enzyme) is the rate limiting step [Luskey and Stephens, 1988]). In the liver the cholesterol is partially degraded into primary bile acids, cholic acid and chenodeoxycholic acid. The bile acids solubilize the cholesterol then the cholesterol can be excreted.

1.1.9. Enterohepatic Circulation.

This is a process in which cholesterol and bile acids circulate continuously between the intestine and the liver.

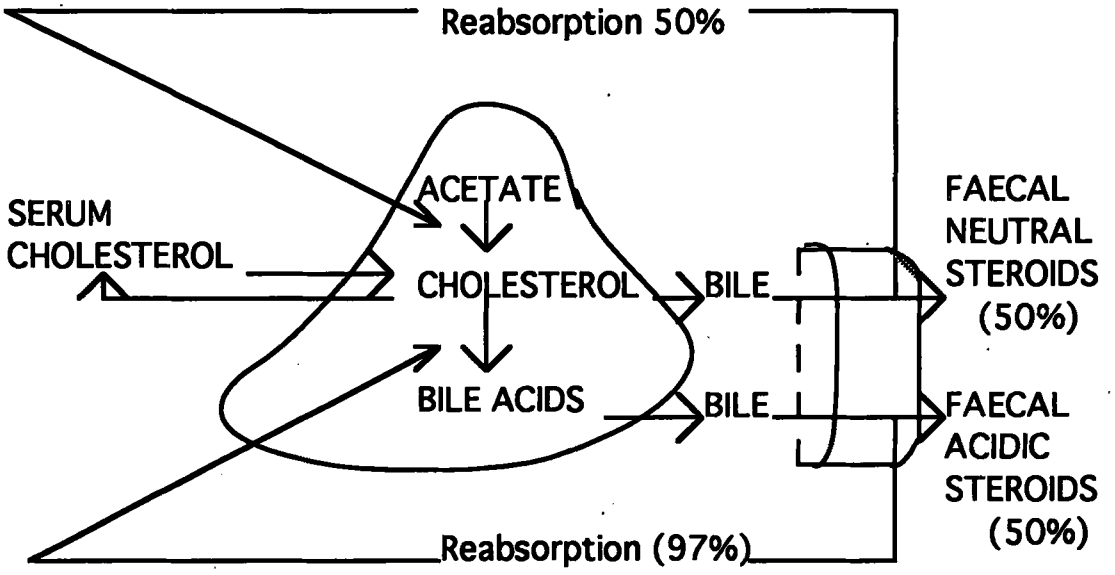


Diagram taken from Cholesterol and Atherosclerosis Diagnosis and Treatment by Scott M Grundy.

High return of cholesterol to the liver suppresses the activity of HMGCoA reductase and therefore inhibits cholesterol synthesis. In contrast low return of cholesterol to the liver results in increased cholesterol synthesis.

Return of bile acids exerts a negative feedback on bile synthesis. In most adults approximately 300-500mg of cholesterol are converted into bile salts each day. The bile salts are then secreted into bile to complete the entero-hepatic circulation. This process occurs on average six times a day.

1.1.10. Cholesterol Transport.

Since cholesterol is highly insoluble in aqueous solutions, it is transported in the lipoproteins, (apolipoproteins), which contain both lipids and proteins.

1.1.11. Lipoproteins.

Lipoproteins are stable, high molecular weight complexes composed of a neutral lipid core (cholesterol esters and triglyceride) and a surface coat of polar lipid (unesterified cholesterol and phospholipids) plus specific proteins known as apolipoproteins (review Assman, 1982). Thus, the polar surface makes it possible to transport the highly insoluble cholesterol esters and triglycerides in plasma. The apolipoproteins on the surface serve several important functions:-

1. Synthesis and secretion of specific lipoproteins.
2. Stabilisation of the surface coat of lipoproteins.
3. Cofactors in the activation of enzymes that modify the lipoproteins.
4. Interaction with specific cell surface receptors that remove lipoproteins from circulation.

Lipoproteins can be divided into several classes based on:- (see Figure 1.3)

1. Density as measured by ultra centrifugation.
2. Electrophoretic behaviour.
3. Apolipoprotein composition.

The main lipoprotein classes isolated by ultra centrifugation from human plasma are; chylomicrons (CM) and very low density lipoproteins (VLDL) both of which are large triacylglycerol rich-lipoproteins synthesised in the intestine and liver respectively. Low

	CM	VLDL	IDL	LDL	HDL_2	HDL_3
Electrophoretic mobility	origin	pre-beta	pre-beta	beta	alpha	alpha
Solvent density for isolation (g/ml)	<0.95	<1.006	1.006-1.019	1.019-1.063	1.063-1.125	1.125-1.210
Molecular weight	$>0.4x10^9$	$5-10x10^6$	$3.9-4.8x10^6$	$2.7x10^6$	$3.9x10^5$	$1.9x10^5$
Diameter (nm)	>80	30-80	25-30	19-25	8-11	6-9
Major apolipoproteins	B-48, AI A-IV, E, Cs	B-100 E, Cs	B-100 E, C	B-100	A-I, A-II E, Cs	A-I, A-II
Composition (% Relative Weight)						
Protein	1-2	6-10	12-16	20-25	35-40	45-55
Phospholipid	2-8	12-18	15-22	20-25	30-40	25-35
Free cholesterol	1	5-8	7-11	6-10	4-6	1-3
Cholestryl ester	1-3	8-14	20-35	35-45	15-20	10-18
Triglyceride	90-96	50-65	25-40	6-12	3-8	3-6

Classification and properties of human plasma lipoproteins.

[Deckelbaum 1987]

Figure 1.3

density lipoproteins (LDL) are major cholesterol-carrying lipoprotein in human plasma. Intermediate density lipoprotein (IDL) has a size and chemical composition intermediate to those of VLDL and LDL. The two major subclasses of high density lipoprotein fractions are HDL_2 and HDL_3 which carry the majority of plasma phospholipids. Lp(a) is a lipoprotein with a density intermediate to that of LDL and HDL_2, concentrations of which have been found to be high in several groups of patients with CHD.

1.1.12. Lipoproteins and Atherosclerosis.

As described earlier, raised levels of plasma cholesterol increase the risk of atherosclerosis. However, individual lipoproteins can have differing effects. For example, although 25% of total serum cholesterol is HDL [Assman, 1982], it appears to have a protective function rather than a damaging one. This was demonstrated in the Framingham Heart studies which took place in Framingham Massachusetts. U.S.A. over a period of thirty years. These showed that patients with less than 25mg/dl of HDL, have an eight times greater chance of incidence of CHD, than those with 65mg/dl [Gordon et al., 1977]. These studies are still ongoing.

Approximately 66% of the total serum cholesterol is made up of LDL therefore, it is not surprising that increased LDL levels, increases the incidence of atherosclerosis (Kannel et al., 1971). It should be noted here that the cholesterol which accumulates in the atherosclerotic plaque is mainly derived from lipoproteins [Newman and Zilversmith, 1962, Review Bowyer, 1967], and that the principal atherogenic lipoprotein is LDL has been well established [Goldstein and Brown, 1977.].

1.1.13. Lipid Transport System Formed by the Plasma Lipoproteins.

The lipid transport system can be divided into two main pathways: an exogenous one for cholesterol and triglycerides absorbed from the intestine and an endogenous one for

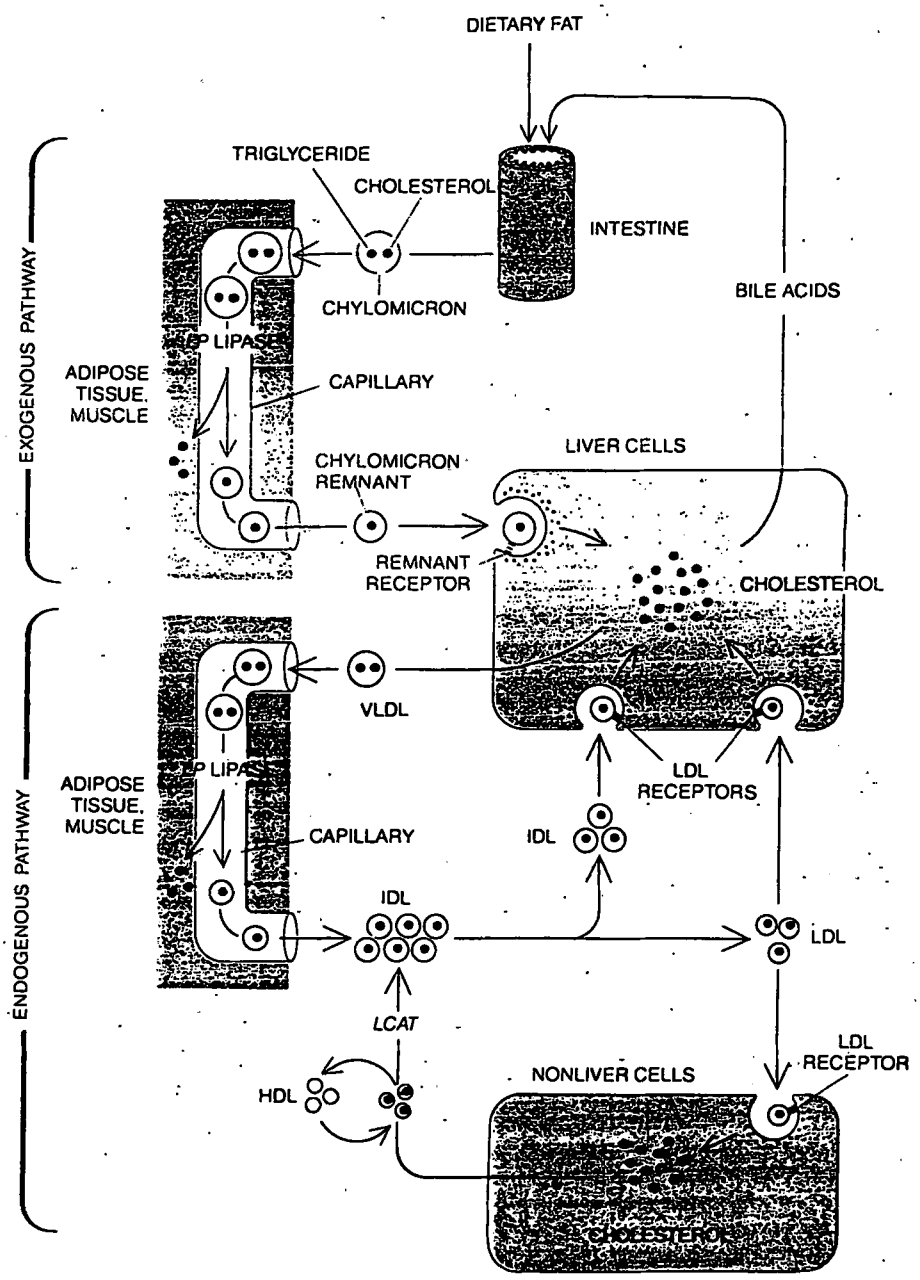


Diagram of the Exogenous and Endogenous Pathways.

[Brown and Goldstein 1984]

Figure 1.4

cholesterol and triglycerides entering the blood stream from the liver and other non intestinal tissues. (See Figure 1.4.).

In the endogenous system, dietary lipids, particularly triglycerides and cholesterol, are incorporated into chylomicrons that enter the bloodstream via the lymph, on the endothelial cell lining of the capillaries. The enzyme lipoprotein lipase (LPL) catabolises part of the triglycerides from the chylomicrons, and this smaller particle retains its apolipoproteins (apo)B and cholesterol ester enriched with apoE. This is now known as a chylomicron remnant and is rapidly taken up by the liver. Here cholesterol can either be secreted back into the intestine after conversion to bile acids or be packaged with triglycerides as very low density lipoproteins (VLDL) particles (which enter the endogenous pathway) containing apoB and apoC as the main apolipoproteins and secreted into the bloodstream. Here the VLDL release some of their triglycerides by the action of LPL in the capillary bed. During this process the VLDL particles increase in density and become enriched in cholesterol ester (from HDL) and apoE. These VLDL remnants, known as IDL, retain their apoB, but lose most of the apoC. The IDL is taken up by the liver by the hepatic LDL receptors. This binding is also mediated by apoE present on the IDL particle. Part of the IDL is converted into LDL thereby losing its apo C and apo E plus most of the remaining triglycerides. In the liver and intestine apolipoprotein-phospholipid complexes are formed, called nascent HDL, which are secreted into the bloodstream. These particles mainly contain apo AI and AII and take up free cholesterol from the peripheral tissue thereby preventing peripheral cells from overloading with cholesterol. The free cholesterol is then esterified by the LCAT enzyme and taken into the core of the HDL particle. Eventually the cholesterol esters of HDL are transferred to VLDL and IDL by the action of cholesterol ester transfer protein.

1.1.14. The LDL Receptor.

In 1974 Brown and Goldstein published the first evidence that the clearance of LDL was mediated by a high affinity receptor. They were investigating the metabolic defects associated with familial hypercholesterolemia and found that HMG-CoA reductase activity in fibroblasts from patients with FH could not be down-regulated by LDL, unlike that of normal subjects (Brown et al., 1973).

The number of receptors displayed on the surface of cells varies with the type of cell and its demand for cholesterol. For example, adrenal glands and ovaries contain sterols cortisone and estradiol, and liver contains bile salts: when the need is low, excess cholesterol accumulates, the cell makes fewer receptors and in consequence LDL is taken up at a reduced rate. Fibroblasts that are actively dividing however, need new membrane material, therefore they maintain a maximum complement of LDL receptors (approximately 40,000 per cell). In fibroblasts that are not growing the cholesterol begins to accumulate, the feedback mechanism suppresses receptor expression and the receptor number may be reduced tenfold. This protects cells against uptake of excess cholesterol, but unfortunately this reduction in receptors means that the rate at which the LDL is removed from the circulation is also reduced, raising the LDL blood level and increasing the risk of atherosclerosis. As mentioned previously the cholesterol in atherosclerotic plaques is derived from lipoproteins, therefore the more LDL in the blood the more rapidly atherosclerosis develops.

The LDL receptor binds the apoB-100 containing lipoproteins such as LDL and apoE-containing lipoproteins such as βVLDL and HDLc [Bersot and Mahley, 1976]. However, four times more LDL particles bind per cell than HDLc suggesting that HDLc binds to 4 receptors [Pitas et al., 1979]. Also Innerarity and Mahley [Innerarity and Mahley 1978] have shown that the affinity for apoE is 10-17 times greater than that of apoB.

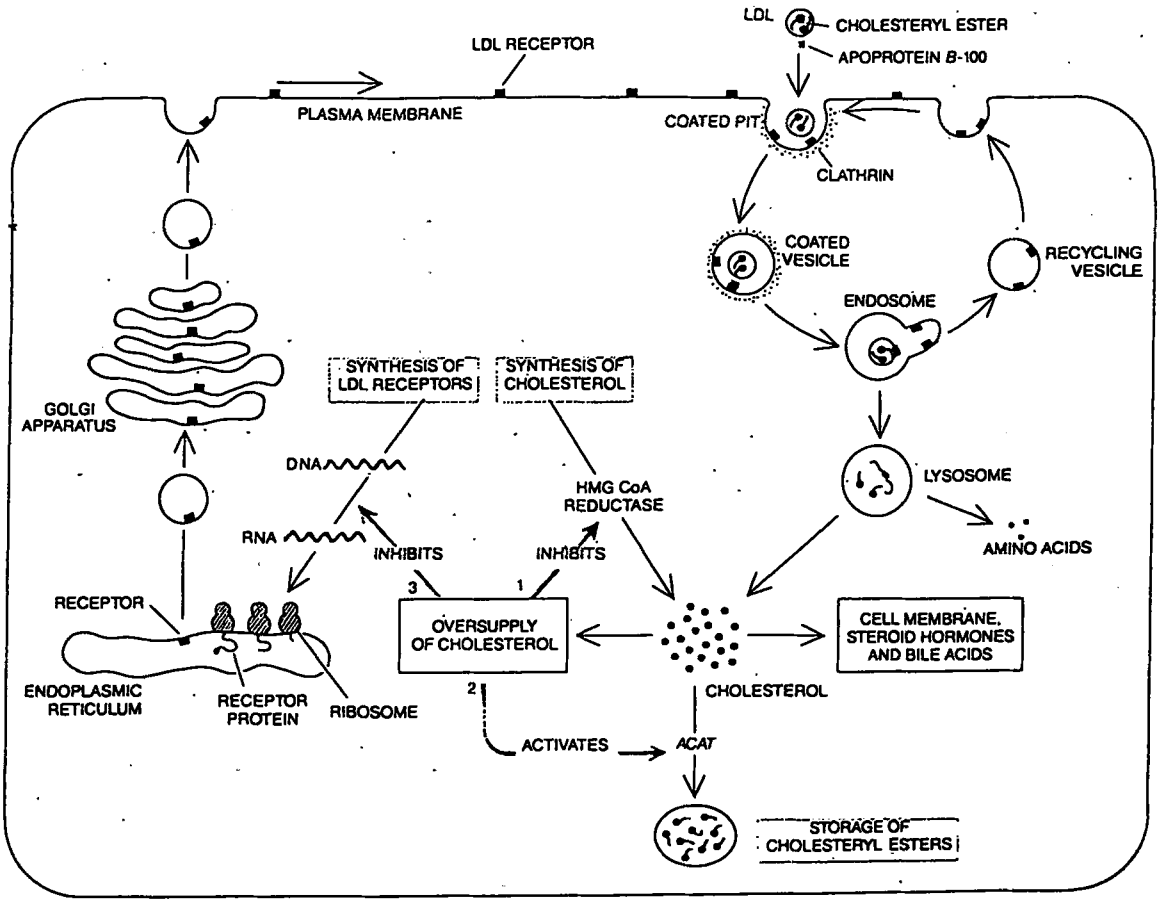


Diagram of cellular cholesterol metabolism; receptor mediated endocytosis of LDL; endogenous synthesis of cholesterol; feedback and regulation of these pathways.

[Brown and Goldstein 1984]

Figure 1.5

The binding of LDL receptor ligands can be disrupted by polyanionic compounds such as sulphated glycosaminoglycans [Goldstein et al., 1976] or by chemical modification of arginine or lysine residues in apoB-100 [Mahley et al., 1979] strongly suggesting that interaction is ionic in nature. The interactions require divalent metal ions, preferably Ca^{2+}, and, thus, the interaction is abolished by the chelating agent EDTA [Goldstein et al., 1977].

1.1.15. How LDL is taken into the Cell (see Figure 1.5).

Initial studies using ^{125}I-LDL demonstrated that LDL not only binds to the cell surface of fibroblasts but is internalised and degraded into its lipids and amino acid components [Goldstein and Brown, 1974]. Subsequent studies show the receptor pathway in more detail [Brown and Goldstein, 1984, Review, 1986,].

Mature LDL receptors are inserted at random in the plasma membrane. In the presence of LDL they are rapidly clustered into clathrin coated pits (Anderson et al., 1976, 1977a and 1977b). The coated pits invaginate to form endocytoic vesicles taking the LDL receptor into the cell. This clustering of the LDL receptors and internalisation takes place whether or not the LDL receptor has bound to its ligand. The vesicles rapidly lose their coat and fuse to form endosomes [Anderson et al., 1977]. Inside the endosomes the pH falls to below pH 6.5 due to the action of the ATP proton pump in the membrane. This pH drop causes the dissociation of LDL from the LDL receptor. The receptor is then recycled back to the cell surface whilst the LDL is delivered to the lysosomes and degraded. The protein is hydrolysed into its constituent amino acids, which are then secreted from the cell, whilst the cholesterol esters are hydrolysed within the lysosomes and then re-esterified for storage elsewhere in the cell.

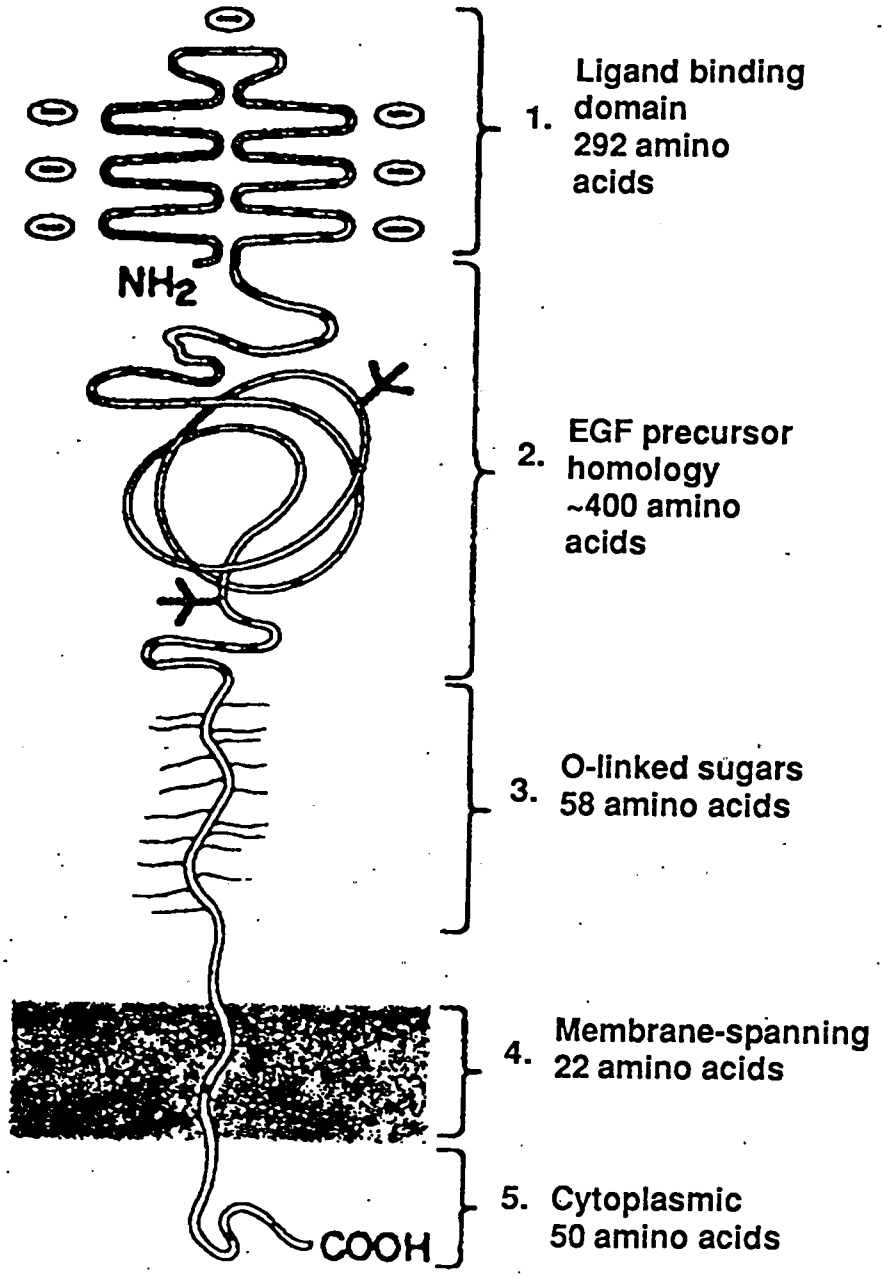
Each LDL receptor undergoes this round trip every 12 minutes. The LDL receptor has a half life of approximately 12 hours thus, the LDL receptor does this on average 100 times before it is degraded [Casciola et al., 1988].

Inside the cell accumulation of LDL modulates three processes:-

1. The ability of the cell to make its own cholesterol is reduced by turning off the synthesis of the enzyme HMG CoA reductase (the rate limiting step in the cholesterol biosynthesis pathway). Suppression leaves the cell dependent on external cholesterol derived from receptor mediated uptake of LDL.
2. Incoming LDL-derived cholesterol promotes the storage of cholesterol in the cell by activating ACAT (acetyl choline acyl transferase). The enzyme reattaches a fatty acid to excess free cholesterol to synthesise cholesterol ester which is deposited as lipid droplets in the cytoplasm.
3. Accumulation of cholesterol within the cell causes a feedback mechanism to inhibit synthesis of new LDL receptors.

1.1.16. **Structure of the LDL Receptor** (see Figure 1.6).

The LDL receptor has a molecular weight of 132000 Daltons and contains 820 amino acids as derived from the cDNA sequence. In addition, it contains a series of carbohydrate moieties. The receptor can be divided into 5 domains each having unique functions. The first domain (amino terminal) has 292 amino acids which contains the ligand binding domain. This region is relatively rich in cysteine residues, all of which are probably involved in disulphide bonds. This region contains 8 repeats of a 40 amino acid sequence which includes a basic region of consensus sequence of Asp-Cys-X-Asp-Gly-Ser-Asp-Glu at the carboxy-terminal end. This may allow it to bind to more than one apolipoprotein at a time and to both apoB and apoE. The second domain of the LDL receptor contains approximately 400 amino acids. This region contains 5 repeats of a 25 amino acid sequence and has a homology to the mouse EGF-precursor protein. This region is thought to have a role in binding LDL, and is required for the acid dependent dissociation of the receptor ligand that occurs in the endosome during receptor recycling



The low density lipoprotein receptor:

A single protein with five domains.

[Brown and Goldstein 1986]

Figure 1.6

[Esser et al., 1988, Davis et al, 1987]. The third domain of the LDL receptor is located immediately outside the membrane spanning region and is relatively rich in serine and threonine and is probably the site where the O-linked carbohydrate chains are added. The fourth domain is the membrane spanning region consisting of 22 amino acids, which are predominantly hydrophobic in nature. The fifth and last domain consists of 50 amino acids at the carboxy-terminus which projects into the cytoplasm of the cell. This region may play an important role in causing the receptors to be clustered into coated pits.

1.1.17. LDL Receptor Deficiency.

Whether LDL receptor deficiency is caused by a genetic defect or is acquired, the end result is the same. VLDL is secreted by the liver and converted to LDL in fat and muscle. In normal people about half of the IDL particles in the circulation are taken up by LDL receptors on the liver cells, the rest is converted into LDL. In people with the genetic defect familial hypercholesterolemia the number of LDL receptors is reduced, which is similar to the deficiency caused by diets that fill liver cells with cholesterol and so reduce receptor synthesis. Both cases give the same result, IDL cannot be taken up by the liver cells and therefore remains in the circulation. The delay in clearance results in a greater conversion of IDL to LDL which in turn further increases the plasma LDL level.

1.1.18. Regulation of LDL (cholesterol levels) by the use of drugs.

70-80% of total LDL catabolism is mediated by the LDL receptor pathway. In vivo the LDL receptor activity is mainly located in the liver; this is most probably due to the fact that in hepatocytes the free cholesterol pool is only partially available for down regulation of LDL receptor activity [Havekes et al., 1987].

In the enterohepatic circulation the liver continuously converts cholesterol into bile; during each cycle only a small fraction (approximately 3%) leaves the body and the rest is reabsorbed. When a resin such as Cholestyramine (non-absorbable bile acid) is ingested the bile acids are prevented from reabsorption in the intestine. Consequently, the

liver uses more cholesterol for bile synthesis, thereby increasing the demand for cholesterol. This results in an increased hepatic LDL receptor activity and a decreased plasma LDL-cholesterol level [Levy et al., 1973]. This usually results in a 15-20% decrease in plasma LDL levels. This level is not more pronounced because, although plasma levels are decreased, the liver increases the activity of HMG CoA reductase, the rate limiting step in cholesterol synthesis, and thus more cholesterol is synthesised [Shepherd et al., 1980].

However, drugs which inhibit HMGCoA reductase such as Compactin and Mevinolin can reduce the plasma LDL cholesterol level in familiar hypercholesterolemia patients by 30% [Mabuchi et al., 1983].

Combined therapy reduces plasma LDL cholesterol levels by up to 50% in familial hypercholesterolemia patients [Mabuchi et al., 1983]. This combination of treatment with a bile acid binding resin and reductase inhibitor, produces a greater reduction of LDL levels, than either type of drug can do on its own.

1.1.19. Effects of hormones on the LDL receptor.

Hypercholesterolemia is often seen in people with hypothyroidism which reduces LDL catabolism by the reduction of the hepatic LDL receptors [Scarabottolo et al., 1986]. *In vitro* the thyroid hormone has been found to affect LDL catabolism by increasing the number of lipoprotein receptors [Chait et al., 1979].

Hyperthyroidism on the other hand increases the number of LDL receptors [Scarabottolo et al., 1986] and diminishes plasma cholesterol levels.

In rabbits 17 α-ethinyl estradiol increases the level of LDL receptor mRNA and a corresponding increase in receptor protein [Ma et al., 1986]. In rats given 17 α-ethinyl estradiol [Hay et al., 1971] LDL receptor activity increased, with a corresponding

increase in lipoprotein clearance from the plasma [Kovanan et al., 1979: Chao et al., 1979].

Insulin and non-insulin dependent forms of diabetes mellitus are associated with raised plasma levels of VLDL, ILDL and LDL [Gibbons, 1986; Chait A et al, 1979,] Wade et al., [Wade et al., 1988] showed that insulin (100u/ml for 48hrs) increased the level of mRNA for LDL receptor two times in HepG2 cells cultured in the absence of lipoproteins, and that insulin overcame the suppressive effects of LDL.

Insulin and T3 were found to increase the specific binding of LDL to monolayers of rat hepatocytes [Salter et al., 1987]. Dexamethasone however, reduced LDL binding. It was found that the effects of the insulin could be seen after one hour, whereas T3 and dexamethasone took between 6-12 hours for a maximum response.

1.1.20. Effect of Diet on the LDL Receptor.

It has been shown in experimental animals that excess dietary cholesterol [Kovanen et al., 1981; Mahley et al., 1981] and triacylgycerols [Spady and Dietschy, 1985] suppress the expression of the LDL receptor in the liver and are associated with large increases in plasma levels of IDL and LDL. Clinical studies have confirmed that the addition of cholesterol [Mistry et al., 1981; Nestel et al., 1982; Packard et al., 1983] or saturated triacylgycerols [Grundy and Ahrens, 1970; Schonfeld et al., 1982] to the diet increases IDL and LDL levels in plasma. LDL turnover studies [Shepherd et al., 1980; Packard et al., 1983] have indicated that these changes in plasma LDL and IDL may be due to a reduced receptor-mediated hepatic uptake of IDL. However, these studies have shown a remarkable individual variation in the response to dietary cholesterol [Mistry et al., 1981]. This indicates that some individuals may suppress hepatic LDL receptors and develop high levels of plasma LDL following a high cholesterol diet, while others maintain normal levels of LDL.

1.1.21. Effects of Age of the LDL receptor.

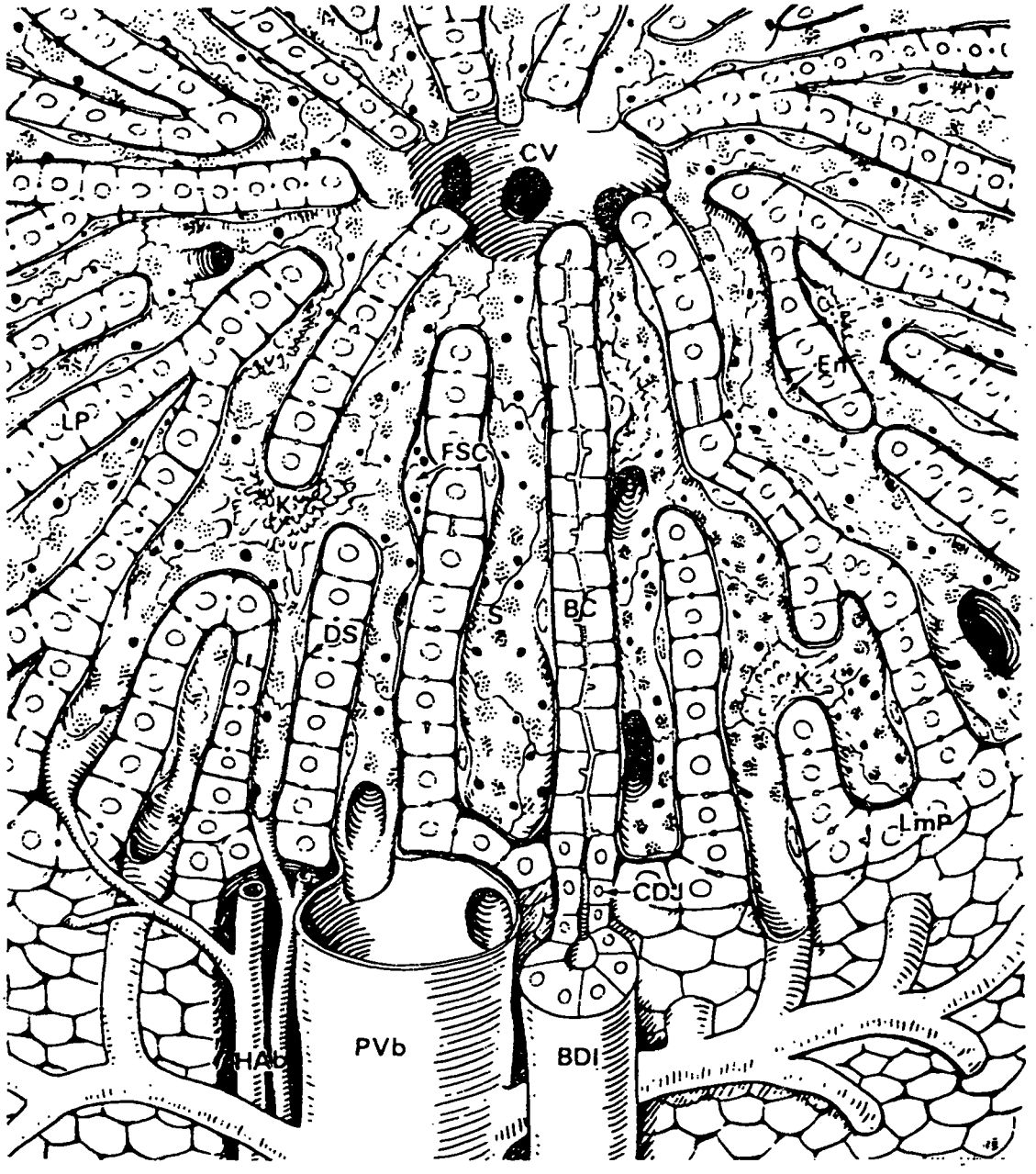
There is direct experimental evidence for the effect of age on the liver LDL receptor [Mahley et al., 1981; Rudling and Peterson, 1985]. It appears that the receptor-dependent, and the receptor-independent catabolism of LDL declines with age in man [Miller, 1984].

1.2. Structure and Function of the Hepatic Parenchyma.

As this research project is primarily concerned with cultured liver cells, it is important to know a little about the structure and function of these cells, together with the role of the extracellular matrix and its relevance in the liver.

The structural and functional unit of the hepatic parenchyma is the acinus (see figure 1.7.). This is organised like a wheel around two distinct vascular beds; six sets of portal triads, each with a portal venule, a hepatic arteriole and a bile duct, form the periphery, and the central vein forms the middle. The parenchyma resemble the spokes of a wheel, and consist of plates of cells, lined on both sides with sinusoidal endothelium. Blood flows from the portal venules and hepatic arterioles at the portal triads through sinusoids that line plates of parenchyma to the central vein. According to the microcirculatory pattern, the acinus is divided into three zones; zone 1 - the portal region; zone 2 - the midacinar region and zone 3 - the region surrounding the central vein.

Hepatocytes and non-parenchymal cells show marked differences, based on their zonal location, in morphology, proliferation and biochemical function. Thus, cells look increasingly larger as they progress from zone 1 to zone 3 and they vary in the morphology of mitochondria, endoplasmic reticulum and glycogen granules. The cells change in shape from zone 1 where they appear diploid, and become increasingly



The liver acinus

The small cells located between the parenchymal plates and the bile ductules are postulated to be the stem cells. The differentiation path can be in one of two directions: towards bile duct or towards hepatocyte.

CV= central vein; K= Kupffer cell; FSC= fat storing cell ; BC= bile canaliculus; En= endothelial cell; S= sinusoid with fenestrations; DS= space of Disse; HAb= hepatic artery branch; PVb= portal vein branch; CDJ= caniculoductular junction (canals of Hering); BDI= bile ductule; LmP= limiting plate; LP= liver plate.

[Reid et al., 1992]

Figure 1.7

polyploid by the time they are in zone 3. Also cells in zone 1 show greater proliferative ability than those in zone 3.

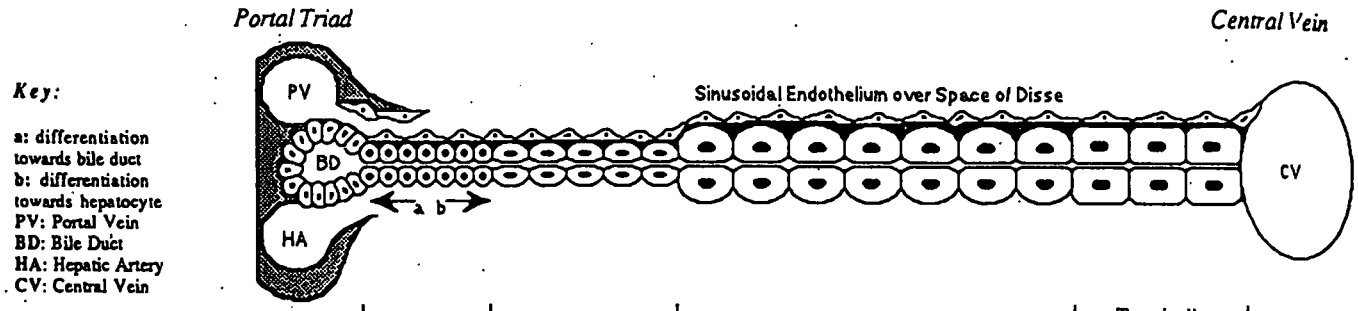
The traditional explanation for the heterogeneity of gene expression in the liver is the changing sinusoidal micro-environment [Campra J. et al., 1982; Traber P.G. et al., 1988; Jungermann K. et al., 1986; Gumucio J.J., 1981]. That is, the liver acinus is perfused unidirectionally from the portal to hepatic venules, so that as the sinusoidal concentration of metabolites changes across the acinus with the uptake and secretion of solutes by hepatocytes. This results in zonal difference in the concentration of oxygen, substrates, cofactors and hormones and thus leads to phenotypic differences in the hepatocyte zones.

Sigal explains[Sigal et al., 1992] the heterogeneity within the liver as a manifestation of a cellular maturation or lineage phenomenon in which stem cells located near the portal triads produce daughter cells which undergo a terminal differentiation process ending at the central vein (see figure 1.8). Thus, cells around the portal triad are considered young cells, whereas those around the central vein are assumed to be terminally differentiated.

The studies done by McGuire [McGuire et al., 1992] to identify the extracellular matrix compounds, and the detailed and extensive ultrastructural immunochemical studies by Martinez-Hernandez and Amenta [Hernandez and Amenta 1992] indicate a gradient in matrix chemistry in the space of Disse from the portal triads to the central vein. This matrix changes not only in amount but also in composition with increasing distance from the canals of Hering (stem cells) and portal triads. There is gradual replacement of basal lamina (type IV collagen, laminin and heparin sulphate proteoglycans) with increasing amounts of fibrillar collagens, fibronectin, chondroitin sulphate and dermatan sulphate proteoglycans. In the region of the central vein the matrix chemistry associated with the parachymal plate is entirely composed of fibrillar collagens, (no laminin) and proteoglycan produced by mesenchymal cells.

Since the matrix is known to 'configure' cells into specific patterns of hormone receptors, ion channels or cell shape, the change in matrix chemistry may alter the responses of cells to hormones, permitting the same hormone or growth factor signals to elicit different biological responses.

Care was therefore taken when working on this project to observe the appearance of the cells whether grown on tissue culture plastic with or without collagen, and Cytodex microcarrier beads with or without collagen, because the surface they are grown on could have altered their morphology and function.



Cell Type	Stem Cells	Transitional Cells	Maturing Parenchyma	Terminally Differentiated Hepatocytes
Cell Size	7-15 μm	15-20 μm	30-40 μm	30-40 μm
Extracellular Matrix (for each category, listed in order of abundance)				
Collagens	IV, V	IV, III	III, VI	III, I, VI
Adhesion Proteins	Laminin	Laminin, Fibronectin	Fibronectin	Fibronectin
Proteoglycans	HS-PG	HS-PG, HP-PG	HP-PG, HS-PG, CS-PG	HP-PG, DS-PG

Model of the liver: Diagram to illustrate the lineage process in the liver with respect to cell size and known variation in the matrix composition.

HS-PG= heparin sulphate PG; HP-PG= heparin PG; CS-PG= chondroitin sulphate PG; DS-PG= dermatan sulphate PG.

[Reid et al., 1992]

Figure 1.8

1.3. Purpose of the Proposed Investigation.

The concentration of low density lipoprotein (LDL) in blood is an important risk factor for cardiovascular disease. The level is determined largely by the activity of the LDL receptor pathway in liver and this project forms part of an effort to study the factors controlling the expression of the receptor.

Most previous studies have so far used the human hepatoma cell line, HepG2, grown in monolayer culture as a model of the human hepatocyte. This system permits the identification of substances that regulate receptor activity, but its application is limited because substances secreted by the cells such as bile acids and LDL itself may cause a feedback regulation of the pathway.

The overall aim of this project was to grow HepG2 cells on microcarrier beads which can then be packed into a column so that substances secreted by the cell can be removed by perfusion . The specific objectives were:-

1. To establish the optimal conditions for the growth of HepG2 cells on microcarrier beads.
2. To develop a column system for perfusion , either single pass (constant fresh medium) or by reperfusion (recycled medium).
3. To find a suitable ligand which would enable the measurement of the LDL receptor on the HepG2 cells in the column system.
4. To determine whether HepG2 cells could be perfused over a twenty-four hour period.
5. To ascertain the effects, if any, on the LDL receptor, on HepG2 cells, caused by single pass perfusion verses reperfusion.
6. To study the maximal and minimal regulation of the LDL receptor in this system by the use of specific drugs.

Chapter 2

GENERAL MATERIALS AND METHODS

2.1. Stock cultures of HepG2 cells.

HepG2 cells were grown in 175cm^2 Falcon flask in 60mls of $RPMI_{1640}$ (flow laboratories) supplemented with 2g/L sodium bicarbonate (BDH), 1.19g/L Hepes (BDH), 0.06g/L penicillin G(Sigma), 0.1g/L streptomycin sulphate (Sigma) and 10% heat inactivated foetal calf serum (Imperial Laboratories) (this complete medium is known as R10) at 37°C in equilibrium with 95% air + 5% CO_2.

The cells were routinely split twice a week at a ratio of 1:6 when they had reached approximately 80% confluence, thus maintaining the cells in a log phase of growth. Briefly, the cells were washed twice with phosphate buffered saline (PBS) to remove any dead or floating cells plus any protein from the medium, then covered with 2mg/ml trypsin in PBS (Flow laboratories) for approximately 2 minutes until the cells started to round up and come off. The trypsinisation was then stopped by the addition of 10mls of R10. The cells were then transferred to a 20ml universal tube and centrifuged for 5 minutes at 1,200 rpm. The supernatant was discarded and the cells re-suspended in 6mls of fresh R10 by sucking up and down with a 1ml Eppendorf pipette 30 times. The cells were left for approximately 30 seconds to allow for any clumps to fall to the bottom of the tube and any debris to float to the top. The 5mls in the middle of the cell suspension was removed and transferred into another tube, re-suspended and placed into five 175cm^2 flasks, then incubated as before. Although the above method may seem tedious, it stopped the cells from growing in clumps, keeping them in a monolayer.

HepG2 cells were used between passage number 91 and 160. A stock of low passage number 78 was obtained from American tissue type collection and stored at -196°C under liquid nitrogen. Freezing the cells was carried out in the following way:- The cells were trypsinised and centrifuged as above, the pellet was then re-suspended in $RPMI_{1640}$

containing 20% heat inactivated foetal calf serum with the addition of 10% v/v DMSO (Sigma) to give approximately 5 x 10^6 cells/ml. The aliquots of 1ml cell suspension were then transferred into sterile 2ml screw-cap vials and frozen overnight at -70°C and finally put into liquid nitrogen at -196°C to store until required.

2.1.1. Plating out HepG2 cells in dishes for assaying.

For plating experiments, the cells were trypsinised, centrifuged and re-suspended as above. This time, after the cell suspension was re-suspended the second time, 100µl of cell suspension was taken out and diluted with 4.9mls of PBS to make a 1:50 dilution and counted using a Fuchs Rosenthal counting chamber. The cells were then plated out at either 150,000 or 300,000 cells per 1ml of R10 + glutamine (made up to a final concentration of 6mM) (known as R10 + Glu) per 1.77cm^2/well in 24 well multiwell dishes (Falcon). The cells were fed on day 1 with R10 + Glu. On day 2 the cells were washed with PBS and re-fed with 10% Bovine foetal calf serum (BLPDS), or medium to be tested. Receptor binding assays were carried out on day 3.

2.1.2. Preparation of microcarrier beads (Cytodex).

Approximately 1g of dry weight Cytodex beads (Sigma) was added to over 50mls of PBS in a glass Scott Duran bottle. This was incubated overnight at 37°C so that the beads could swell to their maximum size and then sterilised by autoclaving at 10lbs/square inch for 30 minutes. The beads were then washed once with a large volume of PBS and transferred into a graduated 50ml sterile centrifuge tube. At this point the beads were allowed to settle, the PBS removed, and an equal volume of $RPMI_{1640}$ to beads was added to make a 50% slurry. This was then stored at 4°C until needed.

2.1.3. Measurement of ^{125}I-LDL binding and internalisation at 37°C on cells grown on tissue culture plastic [based on Goldstein and Brown 1976].

Prior to each experiment, if the cells had been incubated with medium containing LDL extensive washing was required to remove any extracellular LDL. This was done by

removing the medium and replacing it with $RPMI_{1640}$ containing 1% defatted BSA (BDH) and incubated for 20 minutes at 37°C [modification of Havekes et al 1986]. This procedure was repeated three times.

The cells were then incubated with 0.5mls of $RPMI_{1640}$ containing 1% defatted BSA and 5µg/ml of ^{125}I-LDL with a specific activity of 100cpm/ng in the presence or absence of 250µg/ml of unlabelled LDL for 4 hours at 37°C. At the end of this incubation time, the dishes were placed on ice and washed three times for 3 mins on an orbital shaker, speed 4 rpm, with ice cold PBS containing 0.2% BSA followed by one quick wash with PBS only. The cells were then placed at room temperature and incubated with 0.5ml of 1% SDS for 10 mins. The cells were harvested into counting vials, the wells were washed with another 0.5mls of 1% SDS to obtain any remaining lysate. The radioactivity associated with the cells was determined by gamma counting. The cells were then sheared by sucking the cell suspension up and down through a 0.5mm gauge needle with a syringe. A sample was then taken for protein determination, so that ng/mg of cell protein could be found (Lowry method see 2.1.7).

2.1.4. Lysate from cells on beads in columns for protein estimation.

After the LDL receptor assay had been performed on the cells on beads in columns, a protein estimation was required. This was carried out by adding 1ml of 1% SDS to the column (which had been previously sealed) which was then incubated at 37°C for a minimum of 30 minutes. The cells on beads were then subsequently sheared in the usual way by means of a 0.5mm gauge needle attached to a syringe. The beads were then allowed to settle (Cytodex beads interfere with the assay) and a sample of the supernatant was then taken for protein determination by the Lowry method (2.1.7), so that ng/mg of cell protein could be found.

2.1.5. Measurement of ^{125} I-LDL and ^{125} I-rmLDL (reductively methylated LDL) binding and internalisation at 37°C on cells grown on tissue culture plastic.

These experiments were carried out in the same way as for ^{125}I-LDL binding and internalisation at 37°C (see 2.1.3) except that :-

The cells were incubated with $RPMI_{1640}$ containing 1% defatted BSA and 1μg/ml of ^{125}I-LDL (total binding and uptake) or 1μg/ml ^{125}I-rmLDL (non specific binding and uptake).

2.1.6. Measurement of ^{125}I-C7 and ^{125}I-Myb$^{2-3}$ binding and internalisation at 37°C on tissue culture plastic.

These experiments were carried out in the same way as for ^{125}I-LDL binding and internalisation at 37°C (see 2.13) except that:-

The cells were incubated with $RPMI_{1640}$ containing 1% defatted BSA and 3μg/ml ^{125}I-C7 (total binding and uptake) or ^{125}I-Myb$^{2-3}$ (non specific binding and uptake).

2.1.7. Measurement of protein concentration by the Lowry method

Protein concentration of lipoproteins, cell lysate and soluble proteins was carried out using a modification of the Lowry method [Lowry et al., 1951].

Protein determination was carried out in either 10ml glass tubes or in 96 well immuno-assay plates (Nunc). The two methods gave identical protein concentrations for a series of test solutions.

Stock solutions were prepared:

1. 20g/l Na_2CO_3 (BDH), 4g/l NaOH (BDH), 1.6g/l sodium tartrate (BDH) and 10g/l of sodium lauryl sulphate (SDS) (BDH) in de-ionised water and stored at -20°C.
2. 40g/l $CuSO_4$ (BDH) in de-ionised water and stored at room temperature.

3. Bovine serum albumin (BSA) (Sigma) standard at 200mg/l for tube assay or 1.6g/l for plate assays in de-ionised water and stored at -20°C.

Working solutions were prepared freshly by:

4. Adding 1/100 volume of stock (2) to stock (1).
5. Adding 1 volume of Folin-ciocalteau phenol reagent (BDH) to 1 volume of de-ionised water.

For the assay in glass tubes, 0.05 to 0.5mls of BSA standard solutions were pipetted into glass tubes. 0.05ml to 0.5ml of test samples containing 10 to 100µg of protein was then added to the tubes. The volume of both samples and standards was adjusted to 0.5mls with de-ionised water. 2mls of reagent 4 were added, the tubes vortexed and incubated at room temperature for 10 minutes. 0.2ml of reagent 5 was then added, the tubes vortexed, and incubated for 1 hour at room temperature. The absorbence at 750nm was read on a Pye Unicam SP1800 spectro-photometer.

For the assays performed in 96 well dishes, 50µl of de-ionised water was placed in all wells. Doubling dilutions of BSA standard solutions or test samples were prepared by placing 50µl of solution in row A, mixed by sucking up and down with a pipette and then transferring 50µl to row B and so on. 150µl of reagent 4 was then added, the plate mixed on a microshaker (Dynatech) and incubated at room temperature for 10 minutes. 20µl of reagent 5 was added, the plate mixed on a microshaker and incubated for 30 minutes at room temperature. The absorbency was read at 750nm on a Titertek Multiscan plate reader.

2.1.8. Trypan Blue exclusion test.

The Trypan blue exclusion test is a test to find out cell viability, i.e. dead or dying cells let the dye pass through the cell membrane staining it dark blue, whilst live cells exclude the dye allowing them to retain their natural colour.

Procedure:

50µl of cell suspension was added to 250µl of PBS and 100µl of 0.4% Trypan Blue. The cells were then counted, using a haemocytometer and a percentage of dead to live cells was recorded.

2.1.9. Preparation of Low Density Lipoprotein (LDL) from human plasma.

LDL was isolated from freshly prepared human plasma by sequential preparative ultra centrifugation (Havel et al., 1955).

Stock solutions were prepared:

1. Potassium Bromide (BDH).
2. EDTA solution (1) as an anticoagulant; 0.269M Na_2 EDTA (BDH) in distilled water, pH 7.4, filter sterilised and stored at -20°C.
3. EDTA solution (2); 0.2M Na_2 EDTA (BDH) in distilled water, pH 7.4, filter sterilised and stored at -20°C.
4. Antibiotic solutions; Penicillin sodium (PEN-NA from Sigma) 1 x 10^7 1.U/l + Streptomycin sulphate (Sigma) 10g/l in distilled water, pH 7.4, filter sterilised and stored at -20°C.
5. Density salt solution(1) d=1.020g/ml; 1.21g Tris (BDH), 30.6g Potassium Bromide, 5ml of EDTA solution (2), 5ml of antibiotic solution, pH adjusted to pH 7.4 with HCl, to 1 litre distilled water, filter sterilised and stored at -20°C.
6. Density salt solution (2) d = 1.065g/ml; 1.21g Tris 95.9g Potassium Bromide, 5ml of EDTA solution (2), 5ml antibiotic solution, pH adjusted to pH 7.4 with HCl, to 1 litre with distilled water, filter sterilised and stored at -20°C.
7. Density salt (3) d = 1.215g/ml; 1.21g Tris, 241.6g Potassium Bromide, 5ml of EDTA solution (2), 5ml of antibiotic solution, pH adjusted to pH 7.4 with HCl, to 1 litre of distilled water, filter sterilised and stored at -20°C.

8. Dialysis buffer; 0.01M Tris, 0.15M sodium chloride (BDH), 0.001M EDTA in de-ionised water, pH adjusted to pH 7.4.

Blood was collected from the anti-cubital vein of non fasted healthy human donors and placed into 50ml plastic centrifuge tubes containing 500µl of EDTA (1) 0.269M. The tubes were then centrifuged at 2800 rpm for 10 minutes, the plasma taken off, and placed in another centrifuge tube and spun again at 2800 rpm for 10 mins to ensure complete removal of all the red blood cells. The plasma from 4-8 donors was then pooled for each LDL preparation.

For every ml of plasma collected 5µl of EDTA solution 2, 5µl of antibiotic solution and 20.5mg of Potassium Bromide was added to give a density of 1.02 g/ml. The plasma was then placed in pollyallomer quickseal tubes (Beckman) filled to the top with density salt solution 1.02 g/ml and centrifuged at 45,000 rpm for 16 hours at 4°C (55.2 Ti rotor, Beckman L8-M Ultracentrifuge). The top, which contains VLDL and chylomicrons, was taken off, and the infranatant collected. This was then adjusted to a density of 1.065g/ml with 68.9 mg of Potassium Bromide per 1ml of infranatant and centrifuged at 45,000 rpm for 16-18 hours, as before. The top fraction (LDL) was collected using a 0.5mm gauge needle and syringe and dialysed in Visking tubing (Medicell) with three changes of a 50 fold volume excess of dialysis buffer over an 18 hour period at 4°C.

The LDL was filtered through a 0.22µm membrane and stored at 4°C. The concentration of LDL was determined in terms of its protein concentration, as measured by a modification of the Lowry method (see 2.1.7). The LDL was used within four weeks.

2.1.10.Preparation of Lipoprotein Deficient serum (BLPDS) from foetal calf serum by ultra centrifugation (based on Havel et all., 1955).

Stock reagents as per preparation of LDL from human plasma

Foetal calf serum was first heat inactivated at 56°C for 30 minutes in a water bath, and for every 1ml of serum, 5µl of Na_2 EDTA solution and 337mg potassium bromide was added, and then stirred until dissolved. The serum was then placed in pollyallomer quickseal tubes and ultra centrifuged at 45,000 rpm for 24hrs. at 4°C. The top containing the lipoproteins, was then discarded and the infranatant dialysed in Visking tubing (Medicell) with four changes of a 50 fold excess of dialysis buffer over a 24 hr. period.

2.1.11. Reductive methylation of LDL.

Solutions:-

1. Borate buffer; 0.15M sodium tetraborate pH 9.0 containing 0.15M NaCl.
2. Dialysis buffer; 10mM Tris/HCl pH 7.4 containing 0.15M NaCl and 1mM EDTA.
3. Formaldehyde

Method:-

Reductive methylation of LDL was carried out by adding 2ml of borate buffer to 1ml of LDL (2-10mg/ml) in the dialysis buffer and cooled on ice for 30 minutes. The reaction was then started (on ice) by pouring the LDL/borate solution into a tube containing 2mg of sodium borohydride and the addition of 2µl formaldehyde. Further additions of formaldehyde were made at 5 minute intervals for a period of 1 hour, with the solution being transferred, after the first 30 minutes, into a second tube containing 2mg sodium borohydride.

When the reaction was complete the reductively methylated LDL (rm LDL) was passed through a PD10 column equilibrated with dialysis buffer and dialysed over night at 4°C with dialysis buffer. The rmLDL was next filtered through a 0.22µm membrane and

stored at 4°C. The concentration of rmLDL was then determined in terms of its protein concentration, as measured by the Lowry method.

2.1.12. Labelling of Low Density Lipoprotein (LDL), and reductively methylated Low Density lipoprotein (rmLDL), with ^{125}I using the lactoperoxidase method.

LDL was labelled with [^{125}I]-sodium iodide using a method based on that described by Marchalonis and co-workers [Marchalonis et al., 1969].

Solutions were prepared:

1. PBS:- 50mM phoshate buffer pH 7.4 containing 150mM NaCl.
2. Tris/EDTA buffer:-10mM Tris/HCl pH7.4 containing 150mM NaCl and 1mM EDTA
3. PBS / azide:- PBS containing 100mM sodium azide.
4. PBS / KI:- PBS containing 10mM potassium iodide.
5. PBS / peroxide:- 1µl / ml of hydrogen peroxide (30%, Sigma H-1009) in PBS. (Prepare immediately before use).
6. ^{125}I-Iodine:- $Na^{125}I$ solution (Amersham IMS30).
7. Lactoperoxidase:- 1mg/ml of Lactoperoxidase (Sigma L-8257) in PBS. Store frozen in 20µl aliquots.

Method:-

For the iodination of LDL and rm LDL, 1ml of LDL or rmLDL containing 1-5mg of protein was equilibrated in 50mM phosphate buffer by buffer exchange on a PD10 column. Lactoperoxidase (10µl), sodium ^{125}I-Iodine (10µl) was then added to the LDL and the reaction was started by the addition of PBS/peroxide (15µl). The solution was then gently mixed and incubated at room temperature for 15 minutes. The reaction was then terminated by the addition of 100mM sodium azide (15µl) and 10mM potassium iodide (15µl). ^{125}I-LDL was separated from free iodine by size exclusion chromatography in a PD10 column equilibrated in 10mM Tris/HCl buffer containing

1mM EDTA, the eluent collected, in 0.5ml fractions, and the fractions were taken for gamma counting. Fractions containing the protein gamma peak were then pooled, filtered through a 0.22μm filter and stored at 4°C. An aliquot was removed at this stage to determine the protein concentration and specific activity. The specific activity of ^{125}I-LDL was expressed in terms of cpm/ng protein.

2.1.13. Labelling of antibodies with ^{125}I using the lactoperoxidase method.

Antibody was labelled with [^{125}I]-sodium iodide using a method based on that described by Marchalonis and co-workers [Marchalonis et al., 1969].

Solutions:-

1. PBS:- 50mM phosphate buffer pH 7.4 containing 150mM NaCl.
2. PBS / BSA:- PBS containing 1% (w/v) bovine serum albumin (Sigma A-9647).
3. PBS / azide:- PBS containing 100mM sodium azide.
4. PBS / KI:- PBS containing 10mM potassium iodide.
5. PBS / peroxide:- 1μl / ml of hydrogen peroxide (30%, Sigma H-1009) in PBS. (Prepare immediately before use).
6. ^{125}I-Iodine:- Na^{125}I solution (Amersham IMS30).
7. Lactoperoxidase:- 1mg/ml of Lactoperoxidase (Sigma L-8257) in PBS. Store frozen in 20μl aliquots.

Method:-

For iodination of monoclonal antibodies C7 or Myb$^{2-3}$, 10μl of lactoperoxidase and 10μl ^{125}I-iodine was added to 0.5ml of monoclonal antibody in PBS. The reaction was started with the addition of 15μl PBS/peroxidase, then mixed gently and incubated at room temperature for 15 minutes. After this time the reaction was terminated with the addition of 15μl 100mM sodium azide and 15μl of 10mM potassium iodide. The ^{125}I-antibody was then separated from the free iodine by size exclusion chromatography with a PD10

column (which had previously been washed with PBS/BSA and then equilibrated with PBS), and the eluent was collected in 0.5ml fractions. Aliquots from each fraction were taken for gamma counting, then the fraction containing the protein gamma peak was pooled, filtered through a 0.22μm filter and stored at 4°C. An aliquot was then removed to determine protein concentration and specific activity. The specific activity of ^{125}I-antibody was expressed in terms of cpm/ng protein.

2.1.14. Dot blot.

(Effects of glucose concentration on the secretion of apo B by HepG2 cells: Identification of LDL and VLDL)

Stock solutions:-

1. 10mM Tris/HCl pH 7.4, containing 1mM EDTA
2. TBS; 50mM Tris/HCl pH 7.4, containing 150mM NaCl and 2mM Ca_2Cl
3. Milk/TBS; 5% milk powder in TBS.
4. First antibody; TBS containing 20μg/ml rabbit anti-human apo B antibody (Calibochem Cat.no. 17826).
6. Wash solution; TBS containing 0.05% Tween 20.
7. 2nd antibody TBS containing a 1:2500 dilution of HRP conjugated with anti-rabbit IgG polyclonal antibody (Sigma A4914).
8. Diaminobenzidine solution; 100ml TBS containing 50mg 3.3' diaminobenzidine, 50μl 30% H_2O_2, 500μl 350mM nickel chloride ($NiCl_2$).

Method:-

A. Conditioning the medium:-

Six 175cm^2 flasks of the HepG2 cells were grown to confluence in $RPMI_{1640}$ containing 12mM glucose and 10% foetal calf serum. The cells were then washed with PBS and the medium replaced with serum free RPMI containing 2mM, and 20mM glucose (15mls per

flask). After 24 hours the medium was recovered from the three flasks and the debris removed by centrifuging at 500g for 5 minutes.

B. Isolating lipoprotein fractions:-

Potassium bromide (KBr) was added to the medium from each flask to bring the density to 1.020g/ml. 5ml aliquots were ultracentrifuged at 45000rpm, 4°C in a Beckman SW 50.1 rotor for 16 hours, the top 1cm of the solution removed and dialysed extensively against 10mM Tris/HCl pH7.4 containing 1mM EDTA. The dialysed samples were then stored at -70°C. The infranatant was then adjusted to a density of 1.065g/ml with potassium bromide and ultracentrifuged, and the top 1cm of the solution collected and dialysed as before.

C. Dot blot for apo B in lipoprotein fractions from conditioned medium:-

Samples were pre-diluted with TBS in 96 well plates. 100µl aliquots were taken and applied to nitro-cellulose using a BioDot apparatus (BioRad). Dilutions of human LDL (1000ng to 8ng) were used as standards. The nitro-cellulose was then removed from the apparatus and incubated in 5% (w/v) milk powder in TBS for 30 minutes at 37°C, and then for 1 hour at room temperature with the fist antibody (TBS containing 20µg/ml rabbit anti-human apo B polyclonal antibody). After washing three times (2, 3 minute washes and 1, 5 minute wash) with wash solution, the membrane was then incubated with the second antibody (TBS containing a 1:2500 dilution of HRP conjugated anti-rabbit IgG polyclonal antibody) for 30 minutes at room temperature. Finally the membrane was washed as before and the dots visualised using diaminobenzidine solution.

2.2. Statistics.

Unless otherwise indicated data points represent the mean ± the standard error of the mean (sem) of triplicate observations (shown as error bars). In chapter 6 probability studies (P) were also performed on some experiment. When the probability was found to be greater than 0.05, then the results were concluded not to be significantly different from

each other. Interpretation of results less than 0.05 were found to be significant, less than 0.01 were found to be very significant, less than 0.001 were found to be highly significant.

Chapter 3

ESTABLISHING HepG2 CELLS ONTO CYTODEX MICROCARRIER BEADS

3.1. Introduction.

Although in the past hepatocytes have been grown as monolayers on tissue culture plastic, in this project I endeavoured to develop a method of growing the hepatocytes on microcarrier beads. This method should enable perfusion and reperfusion to mimic the environment in the liver and prevent build up of excretory products which may cause an auto regulatory effect resulting in up or down regulation of the LDL receptor. [Havekes L.M. et al., 1986; Carlson T.L. and Kottke B.A. 1989].

3.1.2. Why HepG2 was chosen to study the regulation of hepatic LDL receptor activity.

1. The capacity for studying the individual factors which may influence LDL receptor activity is possible *in vitro*, by varying one factor at a time.
2. A cell line rather than a primary cell culture was chosen, because;-
   a. there is difficulty in getting fresh human biopsies,
   b. the cell line has homogeneity compared with liver biopsies, which may vary from one person to another,
   c. primary hepatocytes rapidly loose their specific phenotype on culturing, shown by their decreased amount of albumin mRNA transcription rate [Clayton and Danell, 1983].
3. Human and not another species of animal must be used to study human LDL receptor activity; this is because different species can have different reactions to the same stimulus. For example, a drop in plasma cholesterol levels was seen in rats given 17α-ethinyl estradiol, [Hay et al., 1971] due to a marked increase in hepatic LDL receptors, with a corresponding increase in lipoprotein clearance from the plasma [Kovanen et al., 1979; Chao et al., 1979]. However Ma et al., [Ma et al., 1986] showed that in rabbit liver 17α-ethinyl estradiol led to a marked

increase in levels of LDL receptor mRNA and a corresponding increase in receptor protein.

For this project a human hepatic cell line known as HepG2 was used. This is an established cell line from a child hepatocellular carcinoma established in 1979 by Aden et al. [Aden et al., 1979] and whose hepatic phenotype was confirmed by Knowles et al., in 1980 [Knowles et al., 1980]. In confluent monolayers, HepG2 cells express normal LDL receptor levels and continue to internalise and metabolise chylomicrons, VLDL, LDL and HDL. Also in lipoprotein-free medium apolipoproteins, AI, AII, AIV, B, C and E are secreted [Thrift et al., 1986] together with cholesterol esters, cholesterol, triglycerides and all the primary bile acids [Javitt et al., 1990].

Havekes showed that HepG2 and primary human hepatocytes were very similar, though not the same in their response to incubation with LDL and HDL, when incubated with increasing amounts of complete normal serum (20 to 100%). In hepatocytes HDL up regulated LDL binding by up to 250%, whereas in HepG2 cells this was shown to be as much as 500% [Havekes et al., 1986]. Hepatocytes show resistance to the suppression of LDL receptor activity by extracellular LDL due to the diversion of cholesterol to cytoplasmic cholesterol esters and external acceptors in preference to the regulatory pool [Havekes et al., 1987]. This feature is also shown by the HepG2 line (Goldstein and Brown). However, HepG2 are incapable of cleaving a side chain off trihydoxcholestanoic acid to produce cholic acid and are unable to conjugate bile acids [Everson and Polokoff, 1986] therefore producing bile salts in a different way to normal hepatocytes.

HepG2 in contrast to Hep3B or PLC-PRF5 [Aden et al., 1979; Twist et al., 1981] cell lines do not produce any hepatitis B antigens or carry any hepatitis B viral DNA sequences, which makes it safer for the handler to work with.

HepG2 cells exhibit features characteristic of the gradation from foetal to adult maturity, (or regeneration to stationary transition) seen in the liver. These changes include a dramatic lengthening or doubling time of the culture at confluence in particular the production of alpha foetal protein (AFP) [Kelly and Darlington, 1989] and a decrease in foetal aldolase and pyruvate kinase, all features of the final stage of the liver development (transition from foetal to adult). There is however, an increase in the adult counterparts; albumin, aldolase B and pyruvate kinase L. The asialoglycoprotein receptors are likewise elevated, therefore making HepG2 cells a good model for the modulation of the liver phenotype which occurs during foetal/adult development or during liver regeneration. However, it must also be noted that HepG2 cells do continue to divide even when confluent in contrast to the mature adult liver which is predominantly non-dividing [Kelly and Darlington, 1989].

In conclusion, HepG2 cells can be said to be a useful model for studying the LDL receptor, but caution must be used before assuming the results will be true *in vivo.*

3.1.3. Cytodex microcarrier beads.

Cytodex microcarriers from Pharmacia are based on a spherical matrix of cross-linked dextran. There are three types of Cytodex microcarrier beads:-

Cytodex 1; cross-linked dextran has been substituted with positively charged N, N-diethyl amino-ethyl (D.E.A.E.) groups though out the entire matrix to a degree which gives optimal cell growth. Cytodex 2; microcarriers are formed by substituting a cross-linked dextran matrix with only a surface layer of positively charged N,N,N, tri methyl-2-hydroxyaminopropyl group. Cytodex 3; has a surface layer of denatured pig skin collagen type I, covalently bounded to a matrix of cross-linked dextran.

Physical characteristics

	Cytodex 1	Cytodex 2	Cytodex 3
Density (g/ml)	1.03	1.04	1.04
Size d_{50} (μm)	180	155	175
d_{5-95}(μm)	131-220	114-198	133-215
Approx. area(cm^2/g dry weight)	6000	5500	4600
Approx. number microcarriers/g dry weight	$6.8x10^6$	$5.8x10^6$	$4.0x10^6$
Swelling (ml/g dry weight in 0.9% NaCl)	18	16	14

NB: Size is based on diameter at 50% of the volume of a sample of microcarriers (d_{50}) or the range between the diameter at 5% and 95% of the volume of a sample of microcarriers (d_5-d_{95}). Information taken from microcarrier cell culture principles and methods by Pharmacia.

3.1.4. Principle of adhesion of cells to culture surface.

Cell adhesion to culture surfaces takes place in four stages whether monolayer or microcarrier techniques are used:-

1. Absorption of attachment factor to the culture surface.
2. Contact between the cell and the surface.
3. Attachment of the cell to the coated surface.
4. Spreading of the attached cells .

Proliferation of anchorage-dependent cells can only occur after adhesion to a suitable culture surface.

This process of adhesion of animal cells to culture surfaces involves cations and glycoproteins, probably fibrinogen found in medium supplemented with serum. The cells come into contact with the surface and attach onto the glycoproteins using synthesised

multivalent heparin sulphate. The attached cells spread and proliferation can then take place.

3.2. Aim.

To find the optimal conditions for establishing HepG2 cells on Cytodex microcarrier beads. Initially for the use in stirrer bottles, but ultimately for the perfusion system.

3.3. Experimental results and Discussion.

3.3.1 Effects of stirring and attachment of HepG2 cells onto microcarrier beads.

3.3.1a. Static cultures.

Preliminary experiments were carried out to find if HepG2 cells would attach to the three different types of microcarrier beads, and if so at what cell density. These seeding conditions were performed in static culture on bacteriological Petri dishes and on glass siliconised dishes. A range of cell to bead densities were examined (i.e. 4mg/ml Cytodex beads to 0.4, 0.8 and 1.6 x 10^6 HepG2 cells/8mls of R10 + Glu). As the cells and beads were not in close contact or in collision with each other, very few cells attached onto the beads, but stayed in clumps.

Further static cultures were performed using bacteriological 96 well plates, so that the cells would be in close contact with the beads and daily re-suspension could take place. Unfortunately under these non-stirring conditions it was found that the HepG2 cells adhered to each other and formed clumps or sheets on top of the beads.

3.3.1b. Stirrer bottles.

It was decided at this point that the cells and beads had to be moving so that they could collide, and hence attach onto the microcarrier beads. However, there were a number of factors which influenced seeding efficiency of the HepG2 cells onto the microcarrier beads in stirrer bottles. These included:

1. The optimisation of HepG2 cells to Cytodex, microcarrier bead ratio.
2. The stirring speed for the initial 3 hours, 24 hours and thereafter.
3. The volume of medium in the initial 3 hours and thereafter.
4. The dimensions of the stirrer bottle.
5. The growth of HepG2 cells on the three types of Cytodex microcarrier beads.

3.3.2. Optimisation of the proportion of HepG2 cells to Cytodex microcarrier beads.

3.3.2a. Density of Cytodex microcarrier beads.

Throughout all these experiments the Cytodex microcarrier bead density remained the same, that is 4mg/ml (final volume) of R10 + Glu. This value was within the range recommended by the manufacturers (Pharmacia) and was high enough to yield quantities of HepG2 covered beads, sufficient for later perfusion experiments.

3.3.2b. Inoculation density of HepG2 cells.

By keeping the Cytodex microcarrier bead density constant only the density of the HepG2 cells was changed.

The initial inoculation density was tested at 3000 cells per cm^2 as suggested in the paper by Visvikis [Visvikis, A., et al., 1990]. This was found to be too low for our needs. Later experiments were carried out at 15,000, 23, 000, 30,000, 60,000 and 90,000 HepG2 cells per cm^2 of bead surface area. (Calculated from the Pharmacia book). Studies were carried out initially by microscopical examination only. These showed that a density of 30,000 and 60,000 cells per cm^2 resulted in approximately 80% confluent beads by day 2. Lower densities than this did not achieve confluence by day 5, and higher than 60,000 produced large amounts of cells/beads clumping together (see plates 1-5). Later LDL receptor binding experiments were carried out, and a cell density of 60,000 per cm^2 was chosen as the optimal cell / bead density, as this appeared to achieve the same value for

Plate 1

15,000 HepG2 cells per cm^2 bead surface area.

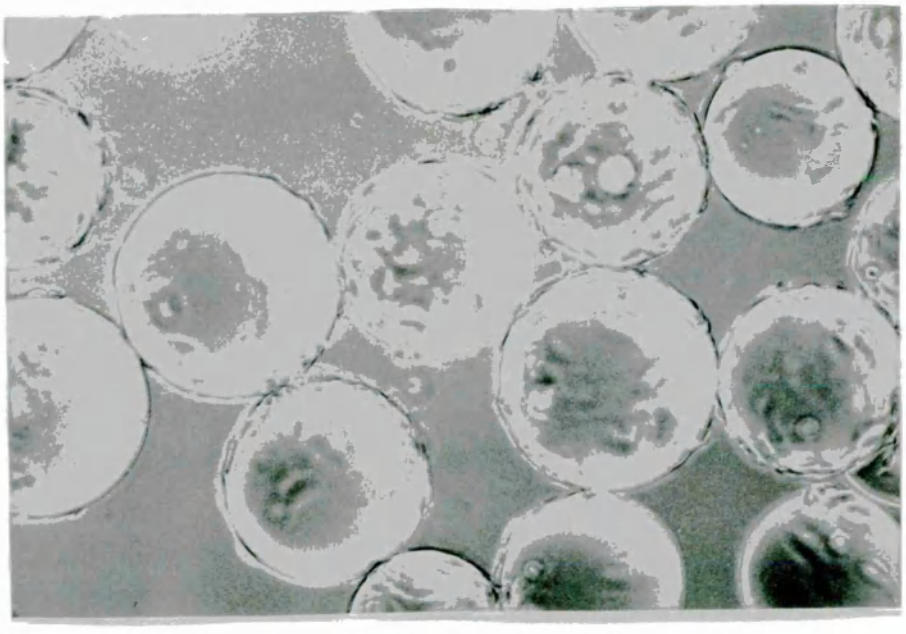


Plate 2

23,000 HepG2 cells per cm^2 bead surface area.

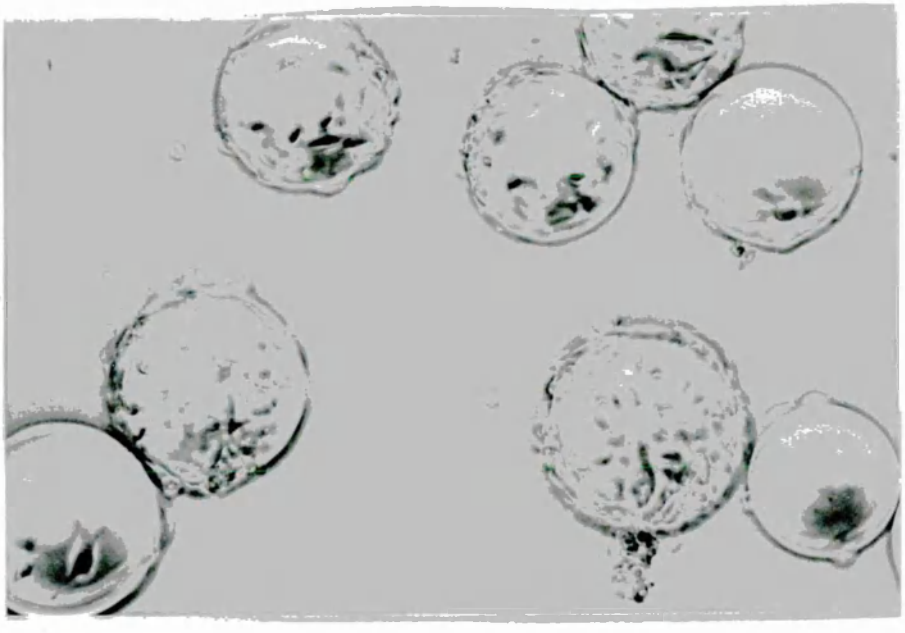


Plate 3

30,000 HepG2 cells per cm^2 bead surface area.

Plate 4

60,000 HepG2 cells per cm^2 bead surface area.

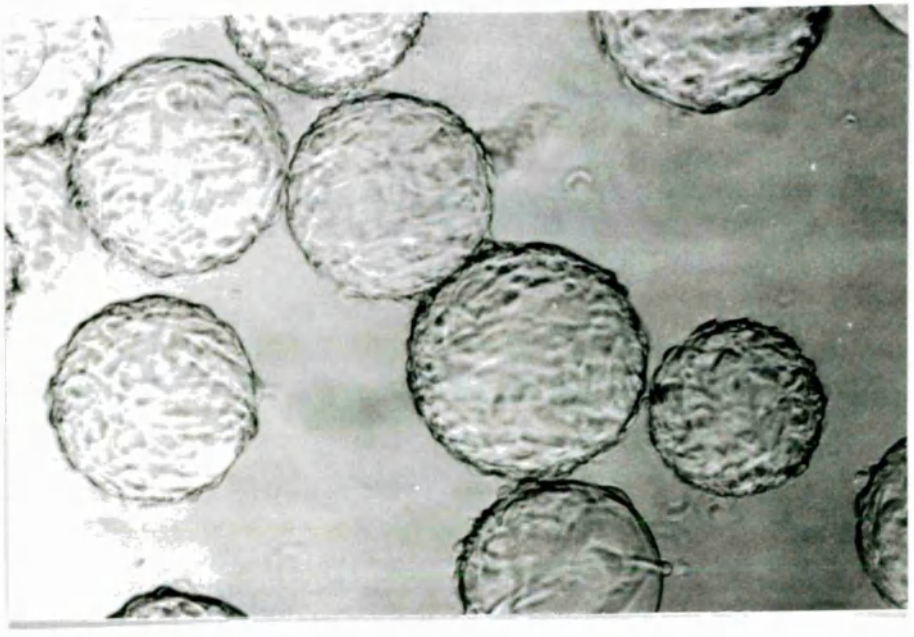
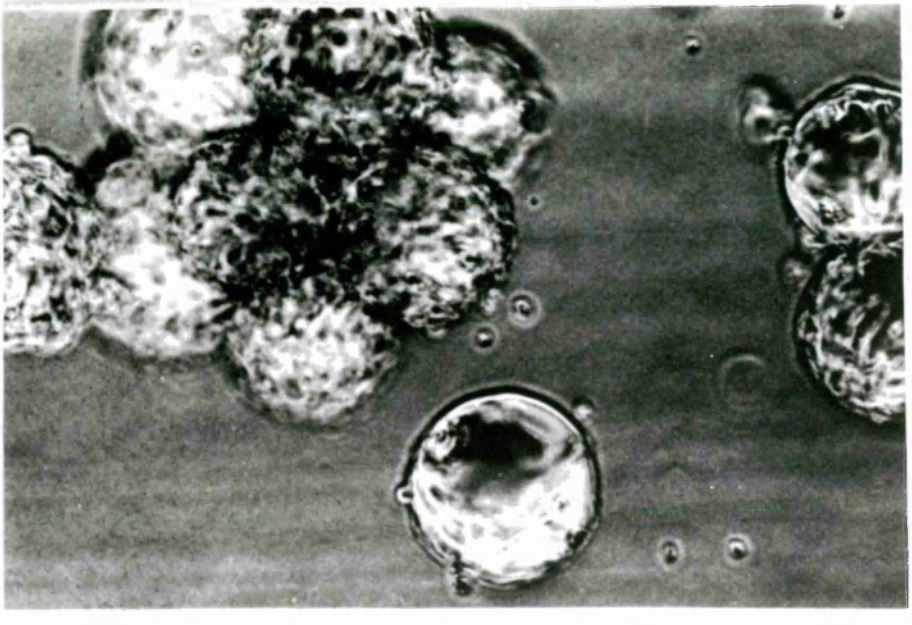
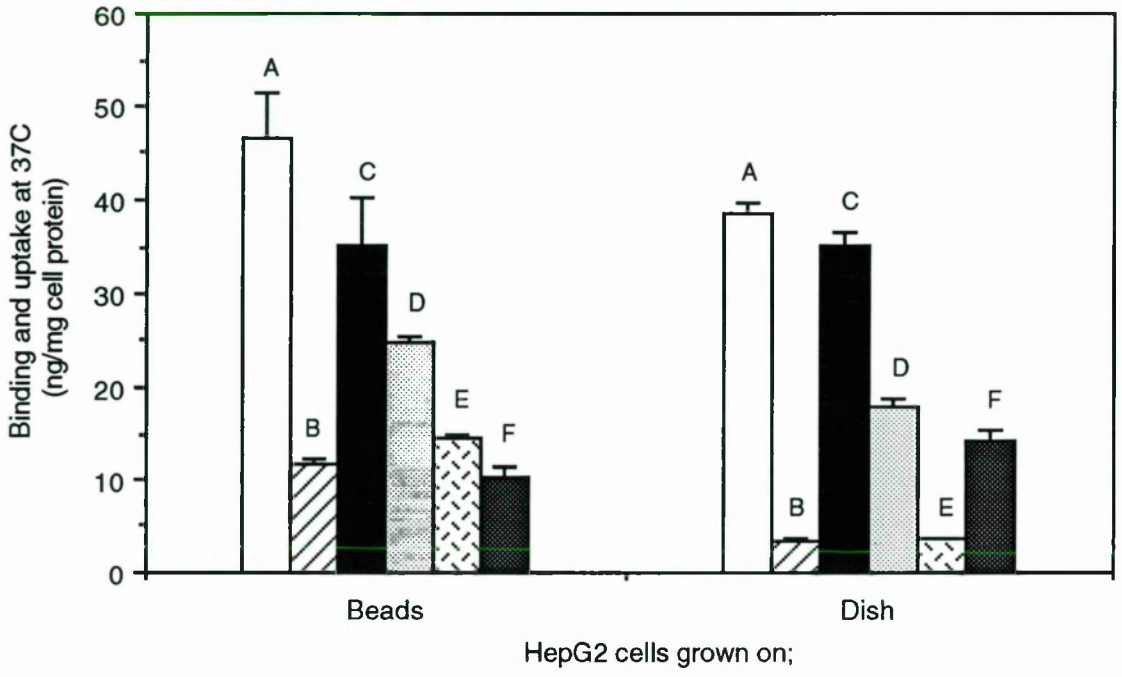


Plate 5

90,000 HepG2 cells per cm^2 bead surface area.





Legend;

A Total binding (BLPDS - 25 hydroxycholesterol)

B Non - specific binding (BLPDS - 25 hydroxycholesterol)

C High affinity binding (BLPDS - 25 hydroxycholesterol)

D Total binding (BLPDS + 25 hydroxycholesterol)

E Non - specific binding (BLPDS + 25 hydroxycholesterol)

F High affinity binding (BLPDS + 25 hydroxycholesterol)

Figure 3.1 Comparison of receptor- mediated binding and uptake of the LDL receptor, on HepG2 cells when grown on microcarrier beads or tissue culture plastic.

Standard method for plating, attachment and pouring 500µl columns in the presence or absence of 25 hydroxycholesterol in BLPDS (see Chapters 4 and 5).

Standard method for plating cells in monolayers and testing in the presence or absence of drugs (see Chapter 2).

Receptor binding assay was performed at 37°C (for 4 hours) using 3µg/ml C7 antibody to measure total binding and uptake. 3µg/ml Myb^{2-3} antibody to measure non-specific binding and uptake. High affinity binding and uptake was calculated by subtracting non-specific from total binding.

Plate 6

Appearance of cells on beads after 24 hours at 20 rpm.

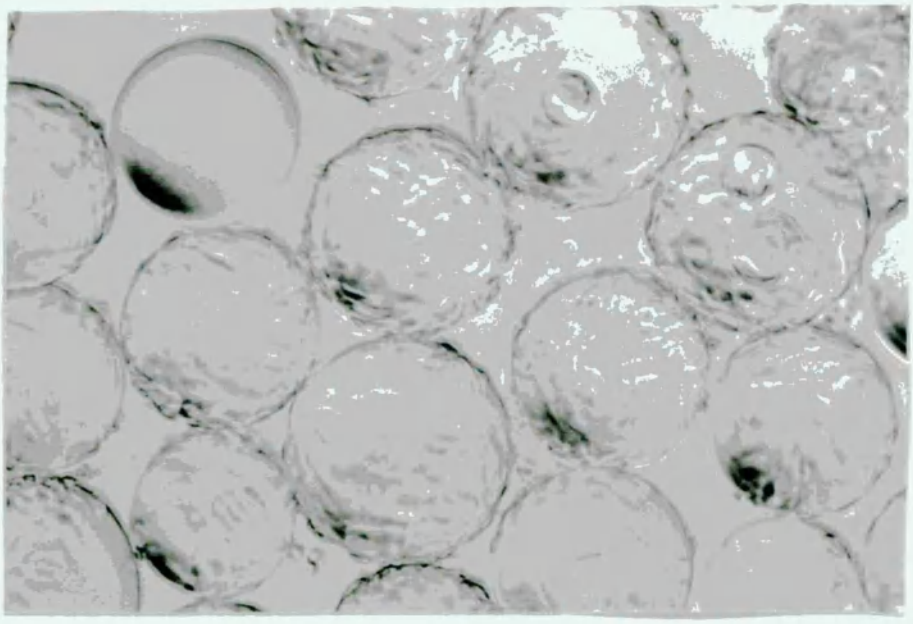


Plate 7

Appearance of cells on beads after 24 hours at 30 rpm

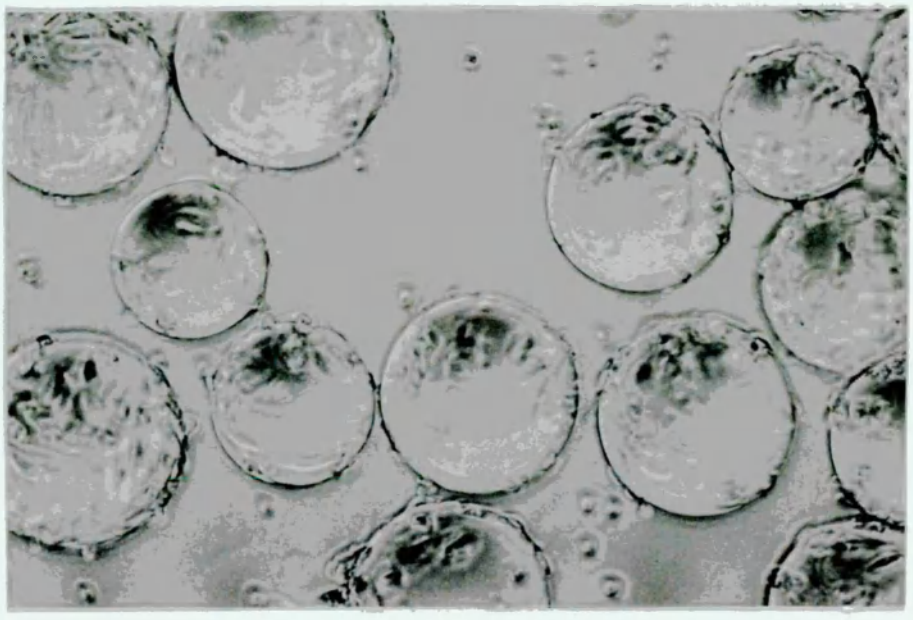
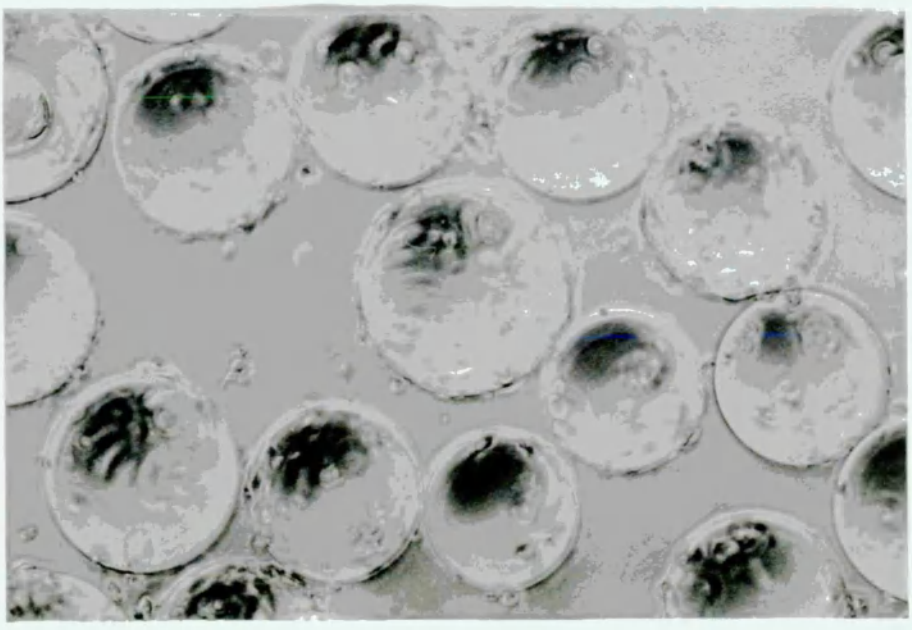


Plate 8

Appearance of cells on beads after 24 hours at 40 rpm.



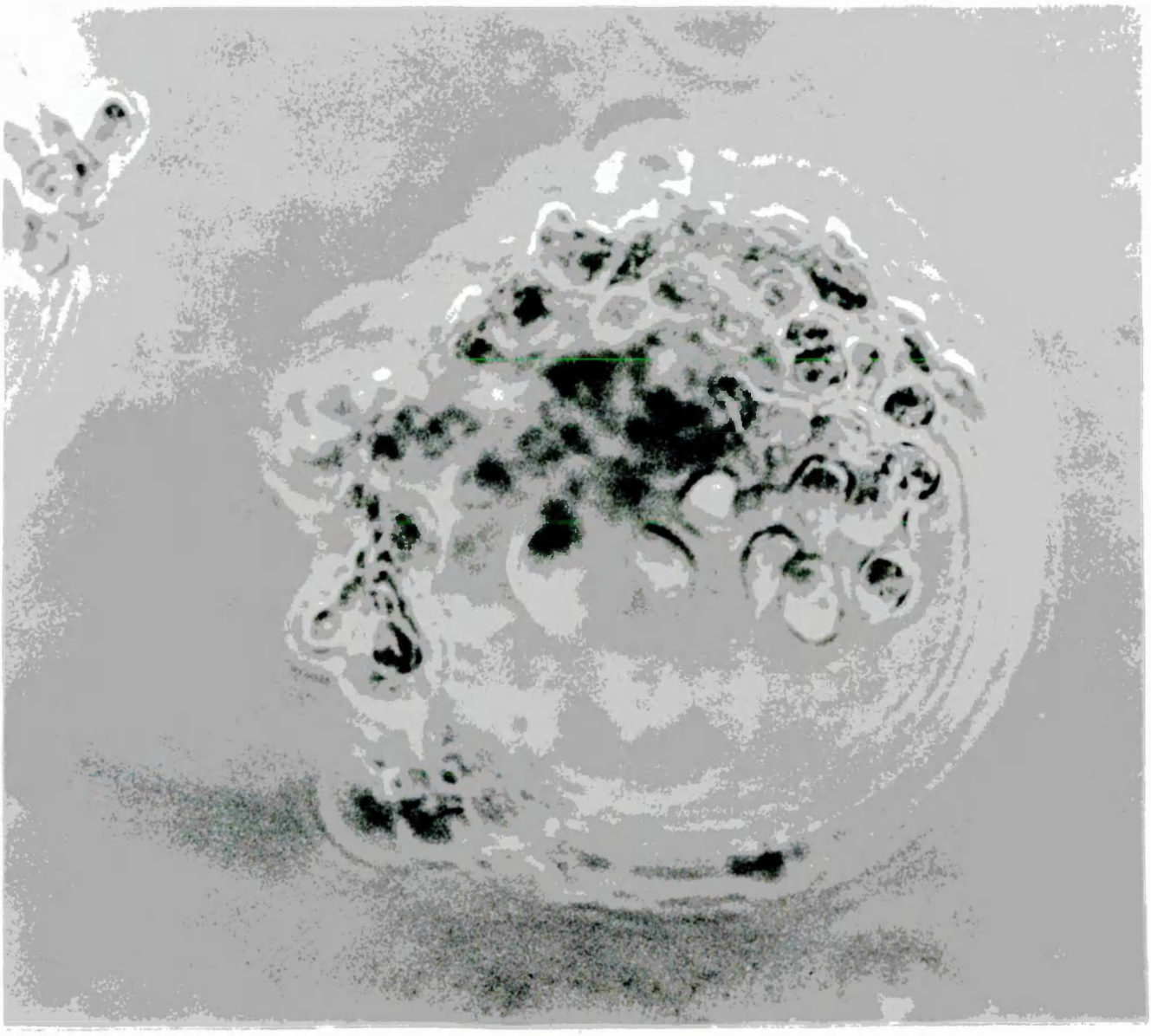


Plate 9

Cell attachment one hour after inoculation

receptor binding per mg cell protein, equivalent to that of cells grown on tissue culture plastic, see figure 3.1.

3.3.3. Stirring speed.

Various experiments were carried out to see the effect of different stirring speeds during cell adhesion in the initial three hours and thereafter. The cell density used was 30,000 per cm^2 bead surface area, and a bead density of 4mg/ml final volume (this in real terms is 33,000,000 HepG2 cells per 200mg (3mls) of hydrated Cytodex 2 beads per 50mls final volume of R10+Glu). This cell density was lower than that later used for LDL receptor assay work, allowing the cells to increase in number/size, rather than be confluent in the shortest period of time.

Half the final volume of medium (R10 + Glu) was used for the first 3 hours (Pharmacia suggested one third, but this volume was insufficient for a 125ml Techne stirrer bottle) and stirred for 2 minutes every 30 minutes of the first 3 hours. Unfortunately all the cells adhered to the bottom of the Techne stirrer vessel and virtually none attached to the beads. This problem was overcome by continuous stirring in the initial period. Plates 6-8 show the effect of stirring for the first 24 hours at 20 rpm, 30 rpm and 40 rpm respectively. Microscopical examination showed that 20 rpm was the optimal speed. Observation of the beads during the first hour after inoculation showed that 80% of the beads had cells attached (Plate 9), although it took approximately 3 hours for the cells to start to spread.

After the initial 3 hours the volume was increased to its final volume, but the speed was kept at 20 rpm. Half the volume of medium was replaced after the first 24 hours and the speed increased to 40 rpm. This was found to be necessary to keep the cells/beads in suspension.

3.3.4. Volume of medium.

As stated in 3.2.3 the volume of medium for the first 3 hours was half that of the final volume (and at a slow stirring speed). This was to produce optimal conditions, that is the optimal opportunity for the cells to collide with the beads, attach, spread and then divide. After this initial attachment the cells needed a larger volume in order that further collision did not detach the cells.

3.3.5. Dimensions of the stirrer bottle.

All the experiments carried out were achieved by using a 125ml Techne stirrer bottle, employing the same bead, cell inoculation density and volume stated previously in 3.3.3 (i.e. 33,000,000 HepG2 cells per 200mg hydrated Cytodex 2 microcarrier beads per 50mls R10 + Glu). The same results were obtained keeping these proportions for volumes from 40mls to 125mls. However, these proportions at these speeds could not be achieved in larger Techne stirrer bottles. This could be due to shear rate etc., but for practical experiments required for this project larger volumes of cells/beads were not required. Therefore, further experiments on this were not carried out.

3.3.6. Growth of HepG2 cells on various types of Cytodex microcarriers.

HepG2 cells were grown on the three types of Cytodex microcarriers i.e..

Cytodex 1:———— positively charged throughout the dextran matrix.

Cytodex 2:———— positively charged on the surface only.

Cytodex 3:———— denatured pig skin collagen type one covalently bounded to the surface matrix (more details see 3.1.3).

Experiments were conducted to find conditions that would support attachment and growth and be most comparable to previous experiments carried out using tissue culture plastic. A growth curve over four days was performed by using three 125ml Techne stirrer bottles simultaneously, at a cell density of 30,000 HepG2 cells per cm^2 bead

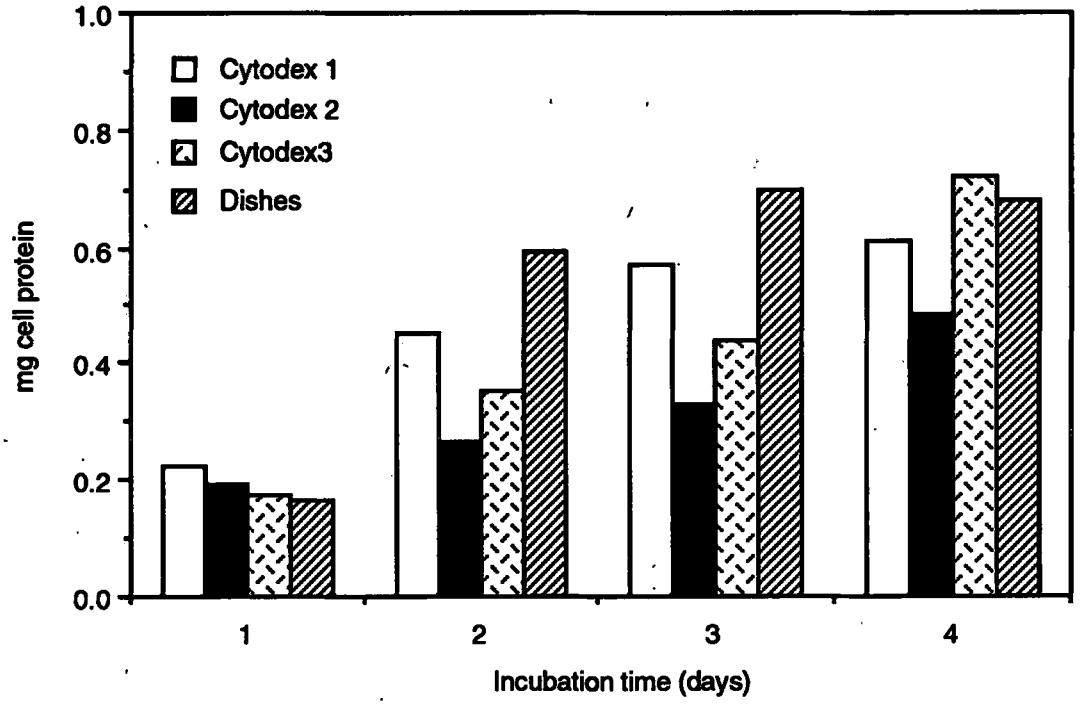


Figure 3.2 Growth curve of HepG2 cells on the three types of Cytodex beads, compared with cells grown in dishes over a period of four days.

HepG2 cells were seeded onto the three types of Cytodex beads at a density of 30,000 cells per cm^2 bead surface area Triplicate samples of 100µl cell / beads were then taken each day over a period of four days. These results were then compared to HepG2 cells which had been grown in 1.77cm^2 dishes over the same period of time. The protein assay was performed by the Lowry method as described in Chapter 2.

surface area, medium volume 50mls R10 + Glu, a plate method was also carried out at the same time for comparison (see Chapter 2).

The results shown in graph Figure 3.2. clearly demonstrate that HepG2 cells have increased in cell protein under all four conditions, that is the three types of microcarriers and the cells grown on tissue culture plastic. Although the lowest increase in cell protein appears to be with HepG2 cells grown on Cytodex 2 microcarriers, this is the microcarrier of choice for the following reasons;

1. According to the Pharmacia book some leakage may occur from Cytodex 1 and this may or may not have an effect on the non-specific LDL receptor binding which is performed at the end of each experiment.
2. Cytodex 3 microcarrier beads are coated with pig collagen type I. In the past, in this laboratory, no work has been performed on collagen. Therefore no comparison in results can be made.
3. Cytodex 2 was chosen, as an increase in cell protein was demonstrated, and the results were comparable with previous work done in this laboratory, on cells grown on tissue culture plastic.

Microscopical examination showed that on all the types of beads, by day 2 the cells had become confluent, but after day 4 cells/beads clumped together and began to detach in sheets.

3.4. Cell viability.

The cell viability of HepG2 cells grown on tissue culture plastic and Cytodex microcarriers was found to be approximately 100% using the Trypan blue exclusion test. This is because only live cells stay attached to stirring microcarriers, therefore all cells attached must be assumed to be alive. Cells grown on tissue culture plastic were washed,

before the test commenced, thus enabling any dead or dying cells to have been washed away.

3.5. Handling microcarriers with confluent HepG2 cells.

Great care must be taken at all times when handling microcarrier beads with confluent cells, so that the cells do not detach. Wide bore, preferably siliconised glass pipettes, were found to be suitable, whereas small plastic Eppendorf pipettes appeared to strip the cells off the beads. Therefore, to keep the problem to a minimum, the transfer of cells/ microcarrier beads was, wherever possible, carried out by pouring rather than pipetting.

Chapter 4

DEVELOPMENT OF THE COLUMN SYSTEM FOR CONTINUOUS RECYCLING OR SINGLE PASS PERFUSION.

4.1 Introduction.

This chapter summarises the stages in the designing of a pumped system that could be used for perfusion, over varying time intervals in a 37°C, 5% CO_2 incubator. This system had to fulfil certain requirements which were:-

A. A column which could be poured easily, and would retain the confluent HepG2 cells on beads without blocking.

B. To determine optimal perfusion conditions for single pass perfusion (an open system which enables medium to flow gently through the column so that cells receive continuous fresh medium), and reperfusion (a closed system with medium being continually recycled).

C. The materials chosen for the column plus beads system had to be suitable for an LDL receptor binding and uptake assay. In particular, materials were selected to minimise non-specific binding and variability of the ligands used to probe the LDL receptor.

4.2. Experimental results and discussion.

4.2.1. Column dimensions.

Initial experiments were carried out using 1ml Sherwood medical syringes. These syringes were narrow bore (0.5cm), with a central nozzle, allowing small volumes of Cytodex beads and cells to be tested. The central nozzle allowed the perfused medium to flow through freely and evenly. Unfortunately the narrow bore gave several problems :-

1. When pipetting highly charged positive beads into polypropylene syringes, the beads, and cells plus beads, became attached to the barrel of the syringe making it impossible for accurate volumes to be pipetted.

2. The small syringe size meant that only small volumes of Cytodex beads plus cells could be tested.
3. The reperfusion volume was limited, due to the small syringe size. Increasing this would have involved complicated reservoir systems for storing the medium.
4. Minimal room was available to allow gaseous exchange of the reperfused system.

Because of these limitations, larger syringes were tested and most of the work carried out in this project was achieved using a 2ml polypropylene Sherwood medical syringe with a central nozzle. This size of syringe had the advantage over the 1ml syringe in that:-

1. The bore size was larger, making cells plus beads more easily, and more accurately poured.
2. Larger columns of cells and beads could be tested (0-500µl), giving greater ^{125}I binding of ligand for the receptor, and more accurate reproducible results.
3. Up to 3mls of medium could be recycled for the reperfused system, so that the ratio of cell number: medium volume would be comparable to cells grown in dishes.
4. The larger bore size and head room allowed better gaseous exchange, thus preventing the cells from dying through lack of oxygen and CO_2.

A larger sized column was tried in an attempt to increase the volume of cells plus beads, and medium further. Unfortunately the large bore size gave problems in that the lightweight filter became dislodged when perfusion and washing took place.

4.2.2. Selection of plug for the column system, which produced minimal non-specific binding.

All experiments were carried out using 2ml syringes and ligand binding to the plug was assessed in the following manner:-

2ml. syringes were plugged with various test samples (Columns without cells or Cytodex beads). Each column was then wetted with 2mls of warm $RPMI_{1640}$, and allowed to drain

fully. The column when capped was filled with 0.5mls, 5μg/ml ^{125}I-LDL, and incubated at 37°C, 5% CO_2 for 4 hours. After incubation the caps were removed, and the columns washed with 2mls of 0.2% BSA in PBS, five times. After washing the syringe wings were removed and both ends of the column covered with Para-film (to prevent ^{125}I contamination). The ^{125}I bound to the column was then counted on a LKB Wallac 1280 Ultra gamma counter.

The plugs tested were cotton and polyester fabrics (Robert Sayles Ltd.), glass-fibre wool, and commercial filters of defined pore size from Millipore, Sartorus, and Spectra/Mesh. The filters and materials were cut to size by hand using a 10mm wod punch (MacKays Ltd.).

In figure 4.1 (Non-specific binding of ^{125}I-labelled LDL onto filters, syringe, and tissue culture dishes) shows results obtained from experiments carried out over several weeks (corrected against the value obtained for 2ml column syringe only).

Generally cotton and polyester fabrics gave high non-specific binding values, and consequently poor reproducibility.

The 0.2μm pore range of filters e.g. cellulose acetate, nitro-cellulose, G.S.W.P. and Durapore, gave much lower non specific binding values. The filters themselves possessed two problems in that, 1) they were very difficult to cut without tearing, and 2) due to their small pore size, there was a risk of blockage with waste products and free cells, during perfusion. Therefore for these reasons no more work on this size filters was carried out.

Polypropylene filters gave relatively low non specific binding, and good reproducibility. The filters tested had large enough pore sizes to prevent them from becoming blocked during perfusion.

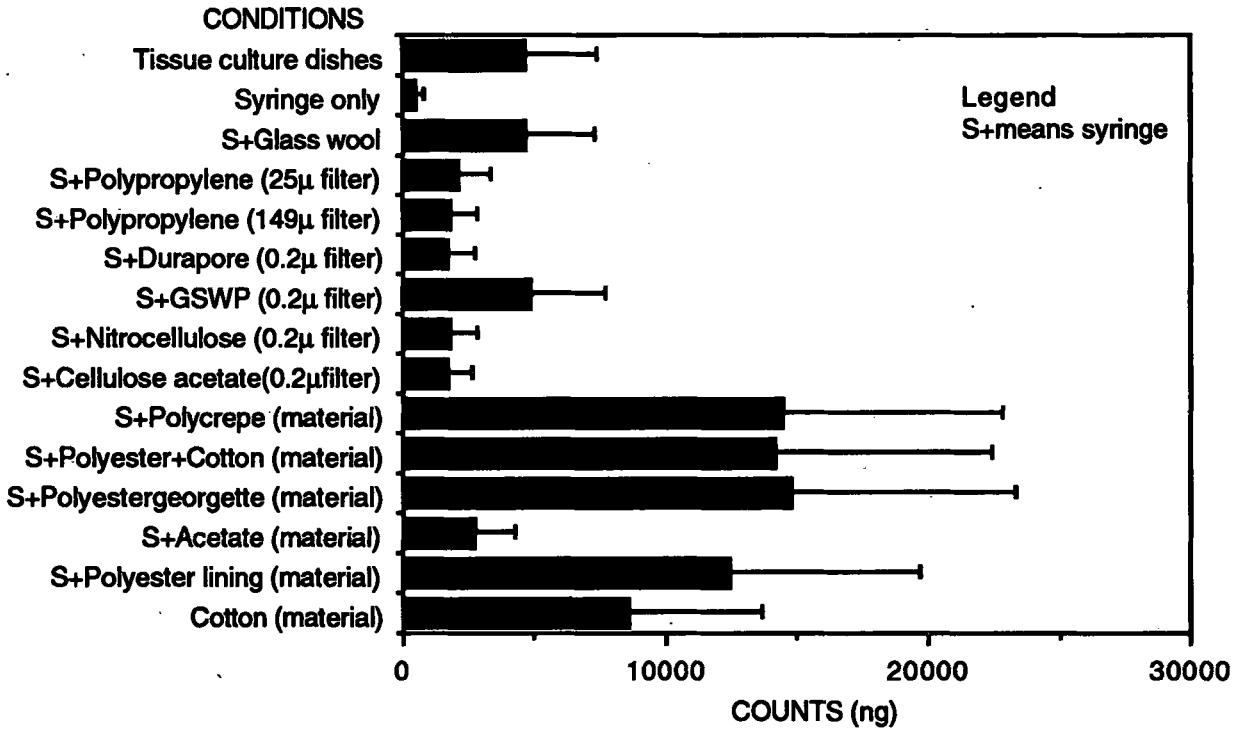


Figure 4.1: Non-specific binding of ^{125}I-labelled LDL onto filters, syringe, and tissue culture dishes.

Results obtained with glass-fibre wool were very variable owing to the difficulties in standardising its density and volume.

Tissue culture dishes, also with no cells, were tested to give a direct comparison. Dishes were incubated with 0.5mls of 5μg/ml ^{125}I-LDL for 4 hours at 37°C 5% CO_2 . Then they were washed four times with 0.5mls 0.1% BSA in PBS, and once with PBS. The ^{125}I-labelled LDL which had become attached to the plastic was removed by treating the dishes with 1% SDS and subsequently counted using the LKB Wallac 1280 Ultra gamma counter. The mean valves and SEM obtained for non specific binding were higher than that of the 2ml barrel syringe and polypropylene filters.

4.2.3. Pouring of HepG2 cells on Cytodex beads into columns.

The HepG2 cells on Cytodex beads in stirrer bottles were allowed to stand for approximately 2-3 minutes. This time was chosen after experiments in which beads were allowed to settle from between 0.5 and 20 minutes. Samples were then taken from the middle of the suspension and the proportion of free cells to confluent beads was assessed. After settling the supernatant was then sucked off, and the cells attached to beads were poured into a 13ml graduated centrifuge tube. The tube was then centrifuged for 10 seconds at 1000 rpm, the supernatant sucked off, and a 50% cell/bead slurry was made with the medium which would be used for perfusion of the columns.

The tube was then gently inverted, and set volumes pipetted into the columns with a 2ml glass pipette. After each 2ml of slurry was pipetted the remaining slurry was again gently inverted. After the columns were poured, set volumes of cell/bead slurry was placed in plastic tubes for a cell count and/or protein assay.

4.2.4. Ratio of Cytodex beads to Cytodex beads with confluent cells.

Different ratios of Cytodex 2 beads were originally tested in the 1ml column syringe system, in an attempt to improve pouring into this narrow bore vessel (percentages tried

were 25, 50, and 75). However the highly positively charged naked beads appeared to stick to the inside bore more easily than the Cytodex beads with cells attached, therefore, the increasing volumes of naked beads meant increasing difficulties in pouring the beads and cell/bead columns.

This experiment was later repeated using a 2ml column syringe. This time the experiment was carried out to try to prevent the cells on beads in columns from becoming clumped, which in consequence could block some of the LDL receptors. However this approach produced no advantages, but two major disadvantages in that:-

1. It was very difficult to distribute evenly naked beads and beads with cells attached, thus creating very variable and therefore very unreliable results.
2. Using naked beads reduced the volume of beads with attached cells that could be tested.

In Chapter 5, which deals with ligand binding, experiments are described which demonstrate that 500µl of cells attached to beads is required to achieve high enough ^{125}I-ligand values, so that up and down regulation of the LDL receptor (e.g. - / + sterol) could be demonstrated.

A 2ml syringe column has a maximum capacity to hold 500µl cells/beads, and 3mls of medium so that sufficient room was available for gaseous exchange. In conclusion no further experiment was carried out as there appeared to be no advantage in doing so.

4.2.5. Development of a pump system.

The first experiments were carried out by allowing the medium to perfuse through the columns by gravity alone. This proved very difficult to standardise, in that even though the columns appeared to be the same, with the reservoir at the same head height, the medium did not flow at the same rate down each column.

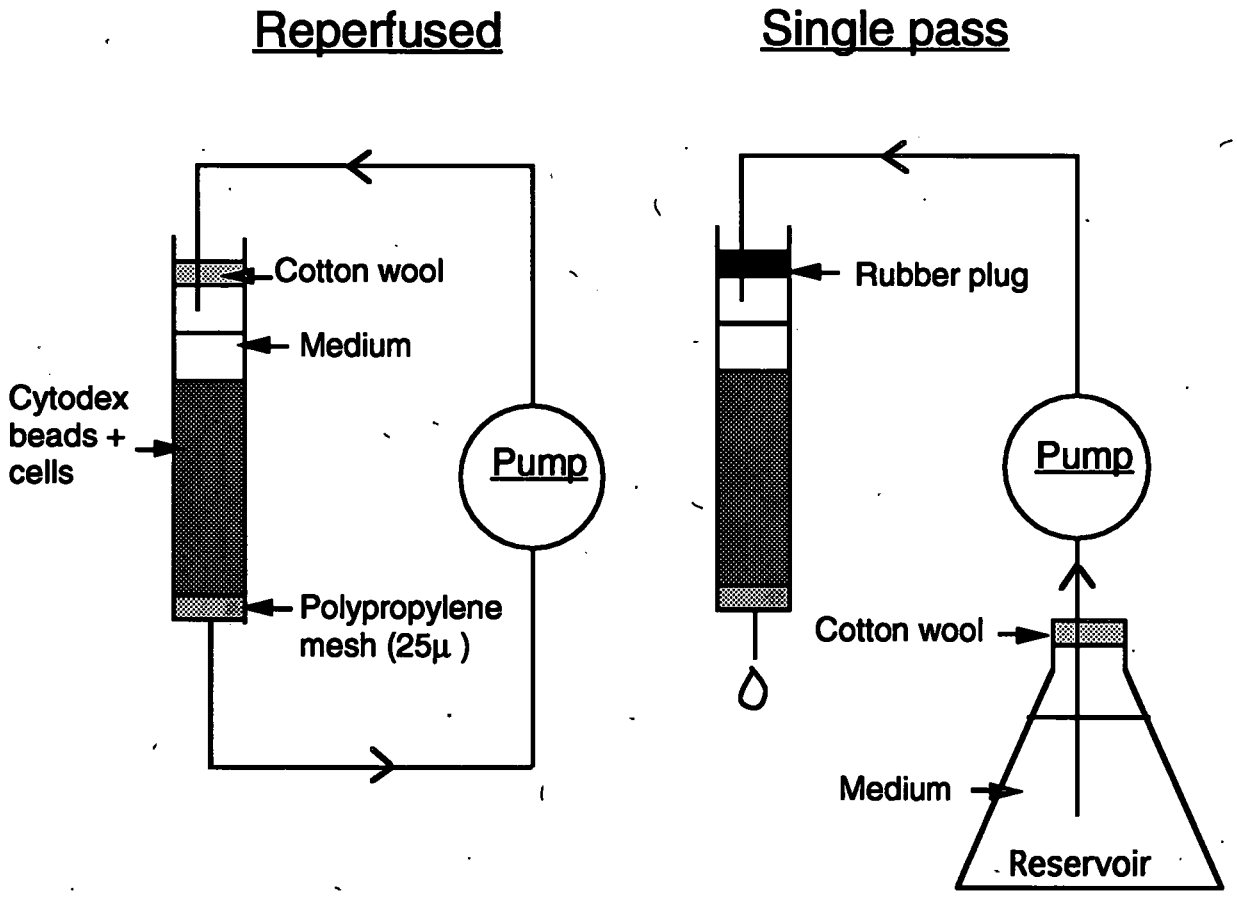


Figure 4.2. Perfusion culture system of HepG2 cells on Cytodex 2 microcarrier beads.

The second series of experiments were carried out using Watson and Marlow peristaltic pump (M HRE 22) with 1mm bore siliconised tubing, which had to be cut to size and pulled taut by hand. This pump system was unsuitable, because the same volume per hour could not be maintained over a 24 hour period.

The third series of experiments were carried out using a Watson and Marlow pump (202U) with 0.8mm silicone tubing. This pump maintained the required flow rate on up to 12 channels over a 24 hour period. The tubing however had to be changed at frequent intervals due to stretching caused by constant pumping at 37°C and sterilisation with 70% ethyl alcohol. Figure 4.2 shows the arrangement for perfusion of HepG2 cells on Cytodex 2 microcarrier beads.

4.2.6. Volume of recycled medium.

The volume used in the recycled perfusion system was decided upon by comparing protein values obtained from cells grown on dishes, ($1.77cm^2$) in 0.5mls of 10% BLPDS, with that of cells on beads in columns and single pass perfused. The protein concentration of cells on beads in columns was approximately five to six times greater than that of cells plated at the same time in dishes. Therefore the calculated volume for reperfusion was 3mls (6x0.5mls).

4.2.7. Pump speed.

The pump speed chosen was one that would give a gentle flow rate, enabling the HepG2 cells to be bathed in medium with minimal turbulence and disruption in the environment of the cells in culture.

Only one speed was tested, this being set at 1ml of medium per hour. This meant that during a 24 hour period the single pass perfusion system would receive 24mls of fresh medium, whilst the reperfusion system would recycle 3mls approximately eight times.

4.3. Summary.

A. Cells attached onto beads in stirrer bottles.

Initially 30mls of medium (10% foetal calf serum (FCS) in $RPMI_{1640}$ with 2g/l $NaHCO_3$, 1.19g/l Hepes, 0.06g/l Penicillin G, 0.1g/l Streptomycin sulphate and 6mM L-Glutamine), 3mls of hydrated Cytodex 2 beads, and 66 x 10^6 HepG2 cells were placed in a stirrer bottle and stirred at 20rpm for 3 hours for attachment to take place. Then the volume was increased to 50mls (see chapter 3 for details).

B. 24 hours later cells were given fresh medium and the stirring speed increased to 40rpm ensuring that the cells on beads were kept in suspension.

C. 500µl of cells on beads were poured into columns in the following way:-

1. The cells on beads in the stirrer bottles were allowed to settle for 3 minutes. Then the supernatant was removed.
2. The cells/beads with any remaining medium was then poured into a 13ml centrifuge tube and spun for 1000rpm for 10 seconds.
3. The medium was removed and fresh medium was added, equal volume to that of cell/beads (the slurry).
4. 1ml of slurry (equal to 500µl cell/beads) was added to each column (2ml syringe with a 25µm polypropylene mesh filter).

D. Attachment of columns to the pump system for single pass perfusion and re-perfusion.

Reperfusion (closed system):- A 19G (Argyle Mediwing) infusion needle was placed cap end onto a 2ml column containing cells on beads. The other end with the needle was cut off, was attached onto the Watson and Marlow 202U pump, and tubing by means of S1 silicone (Portex) tubing. Afterwards the pump system tubing was reconnected to another 19G infusion needle (this time with the cap removed) and placed into the column surrounded by cotton wool so that gaseous exchange with air in the incubator could take place. Prior to the final connection 3ml of medium was added to the column, a constant

speed set i.e. 1ml/hour and run for the number of hours required. (See figure 4.3; which shows a flow diagram from cell attachment to receptor assay).

Single pass perfusion (open system):- The method differed from that used for re-perfusion in that the cap end was placed into a reservoir of fresh medium rather than closing the system, by connection to the column.

E. Finally ^{125}I-Ligand LDL receptor assay could be performed before perfusion and after perfusion so that any change could be observed.

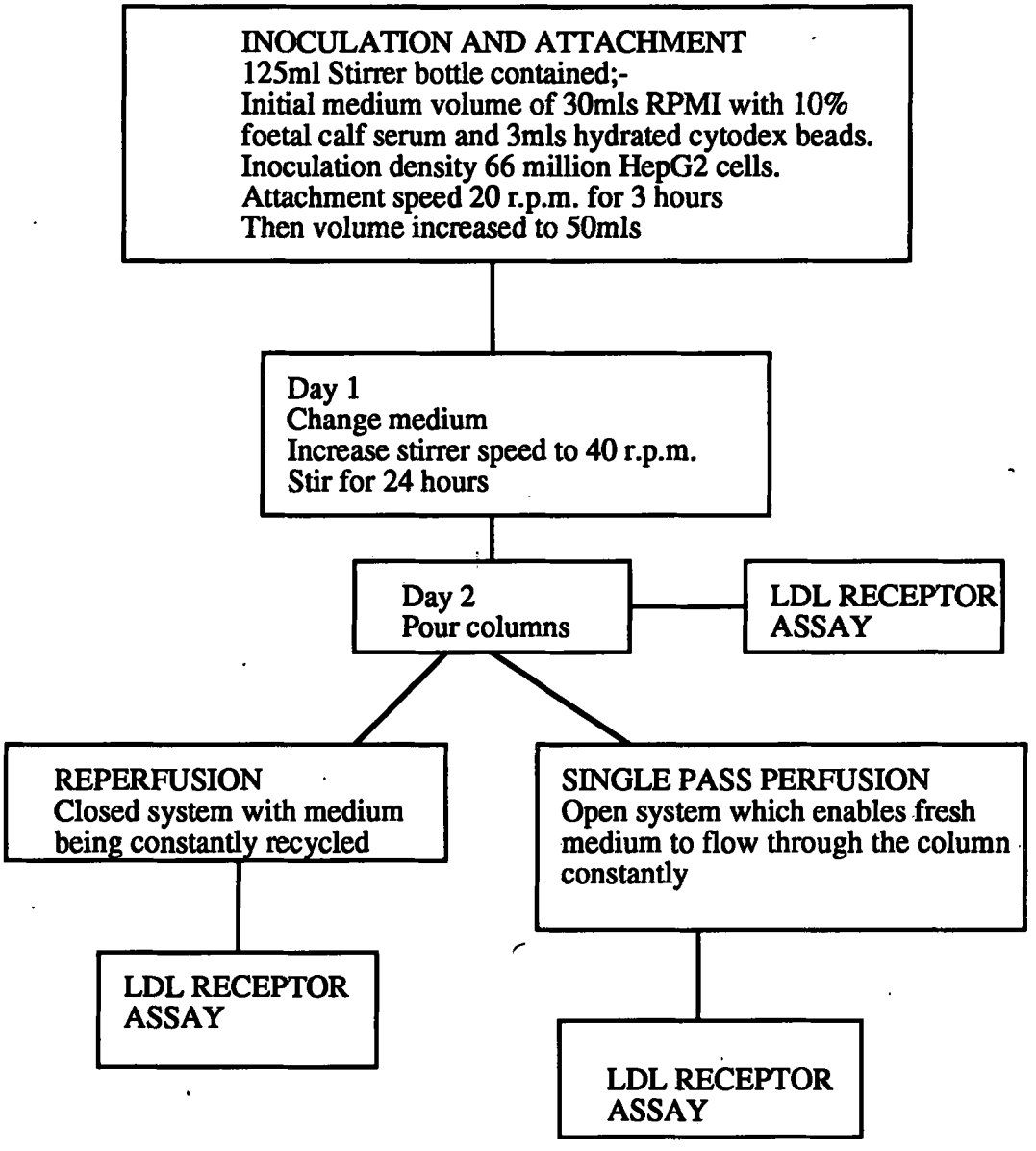


Figure 4.3 Flow diagram from cell attachment to receptor assay

Chapter 5

LIGAND FOR THE MEASUREMENT OF BINDING AND UPTAKE BY THE LDL RECEPTOR

5.1. Introduction.

5.1.1. Low Density Lipoprotein (LDL).

LDL plays a key role in the pathogenesis of atherosclerosis (see Chapter 1). Approximately 65-70% of total cholesterol is transported in LDL, which has a density between 1.019 and 1.063g/ml upon ultracentrifugation. It is composed of 75% lipid (primarily cholesterol esters, cholesterol and phospholipids), and 25% protein, the principal protein being apolipoprotein B100 (which is involved in the binding with the LDL receptor). LDL is predominately derived from the metabolism of VLDL and to a limited extent from the metabolism of chylomicrons.

Catabolism of LDL has been extensively studied by Goldstein and Brown, and many others, who have now demonstrated in tissue culture, and in experimental animals, that there are LDL receptors in virtually all types of mammalian cell. The key function of the LDL receptor however, is concentrated in the liver, where most LDL receptors are expressed. Hepatic LDL receptors help control blood cholesterol concentrations by removing LDL from plasma.

5.1.2. LDL Receptor.

The low density lipoprotein (LDL) receptor is a recycling cell surface protein that mediates the uptake of LDL. The receptor is a single-chain transmembrane glycoprotein with a molecular weight of approximately 132,000 Da and contains 820 amino acids, as determined from the cDNA sequence. The domain structure of the LDL receptor is described in chapter 1. However due to the structural influences which are important to remember to enable an appropriate ligand to be chosen, the five domains are re-described as follows:-

Domain 1 (amino terminal), which has 292 amino acids, is located outside the cell membrane, and is composed of seven copies of an imperfect repeat of a 40 amino acid cysteine-rich sequence [Goldstein, J. L. and Brown, M. S. et al., 1985]. This domain contains binding sites for two proteins, apoprotein (apo) B-100 and apoE, which are components of LDL and VLDL, two lipoprotein ligands for the receptor [Hobbs, H. H., and Russell, D. W., et al., 1986]. Domain 2, has approximately 400 amino acids that are homologous to a sequence precursor for epidermal growth factor (EGF). This is thought to have a role in binding LDL, and is required for the acid dependent dissociation of the ligand that occurs in the endosomes during receptor recycling [Esser, V. et al., 1988, Davis, C. G. et al., 1987]. Domain 3 is relatively rich in serine and threonine and is located immediately outside the membrane spanning region, and it is believed that this region is probably the site where the O - linked carbohydrate chains are added. Domain 4 is the membrane spanning region consisting of 22 amino acids, which is predominately hydrophobic in nature [Goldstein, J. L. and Brown, M. S. et al., 1985; Lehrmen, M. A., and Russell, D. W. et al., 1985; Lehrman, M. A. and Brown, M. S. et al., 1987]. The last domain, domain 5, contains 50 amino acids (carboxy terminus) which project into the cytoplasm of the cell. This region may play an important role in causing the receptors to be clustered into coated pits [Lehrman, M. A. and Schneider, J. L. 1985; Davis, C. G. and Goldstein, J. L. 1986; Davis, C. G. and Goldstein, J. L. 1981].

5.1.3. Receptor recycling.

The LDL receptor is synthesised in the rough endoplasmic reticulum, after which it migrates to the Golgi complex and then to random sites on the plasma membrane. Within minutes it binds to the LDL/ligand and clusters with other receptors in clathrin-coated pits. The receptor-LDL/ligand complex is internalised in coated vesicles, which rapidly shed their clathrin coats and fuse with one another to form vesicles called endosomes. Within the endosomes the receptor and LDL/ligand part company; the receptor returns to the surface and the LDL/ligand is carried to the lysosome, where it is degraded. After

returning to the surface, the receptor binds to another LDL/ligand and initiates another cycle of endocytosis. Each LDL receptor makes one round trip every 12 minutes, or 150 round trips in its 30 hour life span [Brown, M. S. and Goldstein , J. L. et al., 1983].

5.1.4. C7 Antibody.

C7 is a monoclonal antibody, directed against the LDL receptor, and was produced originally by the immunisation of mice with a partially purified receptor preparation from bovine adrenal cortex [Beisiegel et al 1981]. Spleen cells from mice were then fused with the Sp2/0-Ag14 line of mouse myeloma cells. This antibody was thought, by Beisiegel [Beisiegel et al 1981], to bind to the ligand-binding domain of the LDL receptor as 50-70% of bound LDL and VLDL were displaced by C7. Driel et al [Driel et al., 1987] demonstrated that C7 binds to the first cysteine rich repeats in the ligand-binding domain (amino acids 2-42) of the receptor.

5.1.5. Myb^{2-3} Antibody.

Myb^{2-3} monoclonal antibody, which is of the same isotype as C7, was raised against a bacterially expressed protein $bp37^{v\text{-}myb}$ [Evan, G. I. et al 1984]. This monoclonal antibody does not react with the LDL receptor, and is therefore suitable for measurement of non-specific binding and uptake of cells in culture.

5.2. Aims.

This chapter addresses the development of an assay to measure LDL receptor activity in HepG2 cells on beads in columns. This required:-

1. A suitable ligand which would bind specifically to the receptor.
2. A method for correcting for non-specific binding and uptake of the ligand.

Methods tried involved the use of:-

A. ^{125}I-LDL plus ^{125}I-LDL with excess unlabelled LDL.

B. ^{125}I-LDL plus ^{125}I-reductively methylated LDL.

C. ^{125}I-C7 and ^{125}I-Myb^{2-3} (monoclonal antibodies, ^{125}I-C7 which was directed

against the LDL receptor and ^{125}I-Myb$^{2-3}$ directed against an irrelevant antigen which was used as a control for non specific binding).

5.3. Methods.

5.3.1. Standard method.

Any deviation to this will be noted with the appropriate graphs.

5.3.1a. Plating and cell attachment.

Microcarrier beads were coated with HepG2 cells in 125ml Techne stirrer bottles in the following way;-

25mls of R10+Glu (37°C) was placed in each stirrer bottle and 6mls of Cytodex 2 slurry (50/50 beads: R10+Glu) was added. The bottles were then gassed for 2 minutes with 5% CO_2, 95% air and then placed on Techne magnetic stirrers and stirred at 20 rpm, in the 37°C hot room, for a minimum of 15 minutes (this was to allow the beads to be coated with serum proteins so that cell attachment could take place). After this time the stirrer bottles were placed back into the vertical flow cabinet, and HepG2 cells were inoculated at 66 x10^6 cells per bottle. The stirrer bottles were then replaced on the magnetic stirrers and left for a minimum of three hours. The volume of medium in each stirrer bottle was then adjusted to 50mls (final volume) with R10+Glu, regassed and returned to the magnetic stirrer overnight. (Up to a two fold increase of inoculated cells on beads could be produced in this way, provided the same proportions of cells to beads and medium were kept).

The following day the cells on beads were washed with large volumes of warm PBS (approx. 125mls), and then re-fed with 10% bovine foetal lipoprotein deficient serum (BLPDS). The bottles were regassed as before, and returned to the magnetic stirrer.

5.3.1b. Pouring columns.

On day two, the cells on beads were poured into columns (2ml syringe + 25μm polypropylene filter (see Chapter 4)) at a volume of 500μl/column. This was achieved by letting the cells on beads settle in the stirrer bottle for no more then 2-3 minutes (this allowed the cells on beads to settle, but left most of free floating cells still in suspension). The supernatant was then removed and the cells on beads poured into a 13ml Falcon graduated centrifuge tube and spun at 1000rpm for 10 seconds. The supernatant was then removed and an equal volume of medium, either 10%BLPDS or other test medium, added. At this point if more than one stirrer bottle was used, the slurries were gently mixed, so that the slurry was completely uniform.

5.3.1c. LDL receptor assay and/or perfusion.

At this stage six columns could be assayed for binding and uptake of ligand, by the LDL receptor, to obtain a time point zero (see below for details). If required the other columns could be pumped on a Watson and Marlow 202U pump for the required period at 1ml/hour, for either single pass perfusion or reperfusion. At the end of this time the cells on beads could be assayed for binding and uptake of ligand by the LDL receptor.

5.3.1.d. Measurement of ^{125}I-ligand binding and uptake at 37°C in columns.

The columns were placed into an appropriate holder and raised 5-10cm above the surface of the vertical flow cabinet. When the columns had drained they were capped with Nipon stopcocks, which were placed in a closed position, filled with 1ml of medium containing the ^{125}I- ligand, and then incubated at 37°C 5% CO_2 for four hours. Six columns were used for each evaluation, that is, three columns to measure total binding (3μg/ml ^{125}I-LDL, or 3μg/ml C7), and three columns to measure non-specific binding (3μg/ml ^{125}I-LDL+250μg/ml unlabelled LDL, or 3μg/ml reductively methylated LDL (rmLDL), or ^{125}I-Myb $^{2-3}$). At the end of this time the cells on beads in columns were washed, four times with 2mls of 0.2% BSA in PBS (at 37°C), and three times with 2mls of PBS (at 37°C). The columns were then sealed with Parafilm and counted on an LKB 1280 Ultra

gamma counter for one minute. After this the cells on beads were lysed with 1% SDS at 37°C for one hour. The lysate was then sheared six times, by using a 0.8mm gauge needle, and a protein assay performed using the Lowry method.

5.3.2. ^{125}I bound to ligands.

^{125}I was bound on to an appropriate ligand (LDL, reductively methylated LDL (rmLDL), C7 and Myb$^{2-3}$) so that LDL receptor binding and uptake could be measured. This method involved the use of lactoperoxidase in which iodine atoms substituted otho to the hydroxyl group in the phenolic ring of tyrosine [Marchalonis 1969].

5.3.3. Validation of binding assay using ligands on HepG2 cells grown in monolayer culture.

Every time a receptor binding and uptake assay was performed on the columns an assay using cells plated on tissue culture plastic was also carried out in the following manner;- The HepG2 cells were plated out in Falcon 24 multiwell plates (1.77cm^2/well). Each well was seeded with 0.3 x10^6 cells in 1ml R10+Glu. On day one the cells were washed with 2mls of PBS (at 37°C) and re-fed with 500µl of medium containing 10%BLPDS in the presence of 2.5µM 25 hydroxycholesterol to down-regulate LDL-receptor activity, and in the absence of 25 hydroxycholesterol to up-regulate receptor activity. This was to test, in each assay, that the ^{125}I-ligand was working appropriately. The assay itself was performed in the same way as for columns with the exception that the washes were carried out at 4°C instead of 37°C (cold BSA/PBS would have shocked the cells on the beads which would have in turn caused the cells to dissociate from the beads, allowing them to be lost during washing).

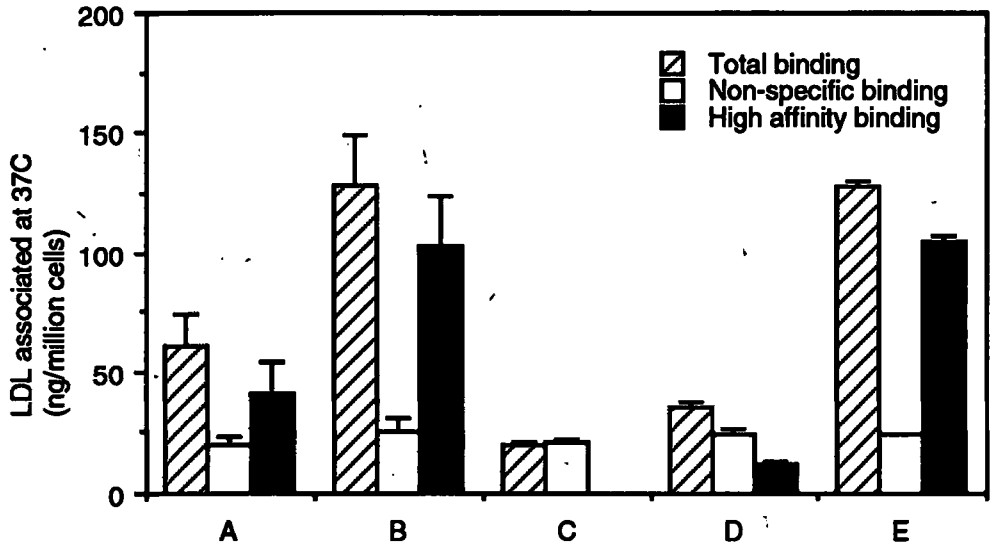
5.3.4. Quality control of ^{125}I labelled antibodies.

In order to minimise variations in binding efficiency as much as possible, each time a new batch of antibody was labelled with ^{125}I, it was tested against ^{125}I labelled LDL in the following way;-

HepG2 cells were plated out in Falcon 24 well plates. Each well was seeded with 0.3 x10^6 cells in 1 ml R10+Glu. On day one the cells were washed with PBS at 37°C and re-fed with 500µl of medium containing 10% BLPDS in the presence or absence of 2.5µM 25 hydroxycholesterol, in the same way as for controls above. The receptor assay was then performed with ^{125}I-LDL and C7 as described above. The total, non-specific and high affinity (receptor-mediated) binding was calculated, and the results compared.

5.3.5. Statistics.

All data points represent the mean ± the standard error of the mean (s.e.m.) in triplicate observations.



Legend

A Column + naked Cytodex 2 beads

B Column + Cytodex 2 beads with HepG2 cells

C Cytodex 2 beads

D Cytodex 2 beads with HepG2 cells

E Mono-layer HepG2 cells grown on tissue culture plastic

Figure 5.1 LDL receptor activity measured by using ^{125}I-LDL, and ^{125}I-LDL+excess unlabelled LDL at 37°C.

The standard plating and attachment protocol was utilised for the initial 24 hours, and the medium then changed to 1% defatted BSA instead of 10% BLPDS. A column volume of 100µl HepG2 cells on Cytodex 2 beads was used is this instance.

The assay was performed using 5µg/ml ^{125}I-LDL to measure total binding, 5µg/ml ^{125}I-LDL plus 250µg/ml unlabelled LDL to measure non-specific binding (see 5.3.1d). High affinity binding was calculated by subtracting non-specific binding from total binding. C and D values were obtained by taking the content out of the columns and measuring them in a clean counting vial.

The HepG2 cells plate control was incubated for the last 24 hours in 1% defatted BSA.

5.4. Results and Discussion.

This chapter presents the results of experiments to establish a suitable ligand which would bind specifically to the LDL receptor (binding and uptake at 37°C), with minimal non-specific binding to the column plus beads.

5.4.1. LDL receptor activity measured by using ^{125}I-LDL, and ^{125}I-LDL + excess unlabelled LDL at 37°C.

The first method tried was a standard method for the determination of the LDL receptor activity on cells grown on tissue culture dishes. This involved the use of 5μg/ml ^{125}I-LDL in the presence or absence of a large excess of unlabelled LDL (250μg/ml) [Goldstein J. L. and Brown M. S. et al 1976]. In this method ^{125}I-LDL binds to the LDL receptor, any non-specific binding which may occur by this method is determined by ^{125}I-LDL competing with a fifty fold excess of unlabelled LDL. High affinity or receptor mediated binding is then calculated by subtracting non-specific from total binding.

In Figure 5.1 shown opposite, which involved the use of columns plus Cytodex beads in the presence or absence of HepG2 cells, shows that Cytodex 2 beads with HepG2 cells attached (B), assayed in this way, exhibited significant high affinity binding, which was comparable to HepG2 cells grown in mono-layer, on tissue culture plastic (E). However, it could also be clearly seen that some pseudo - specific (high affinity) binding is produced by the naked Cytodex 2 beads in columns (A), and that this is due to the columns and not the Cytodex beads themselves. This could be due to the LDL being sticky, and therefore sticking to the columns with high affinity, mimicking specific binding.

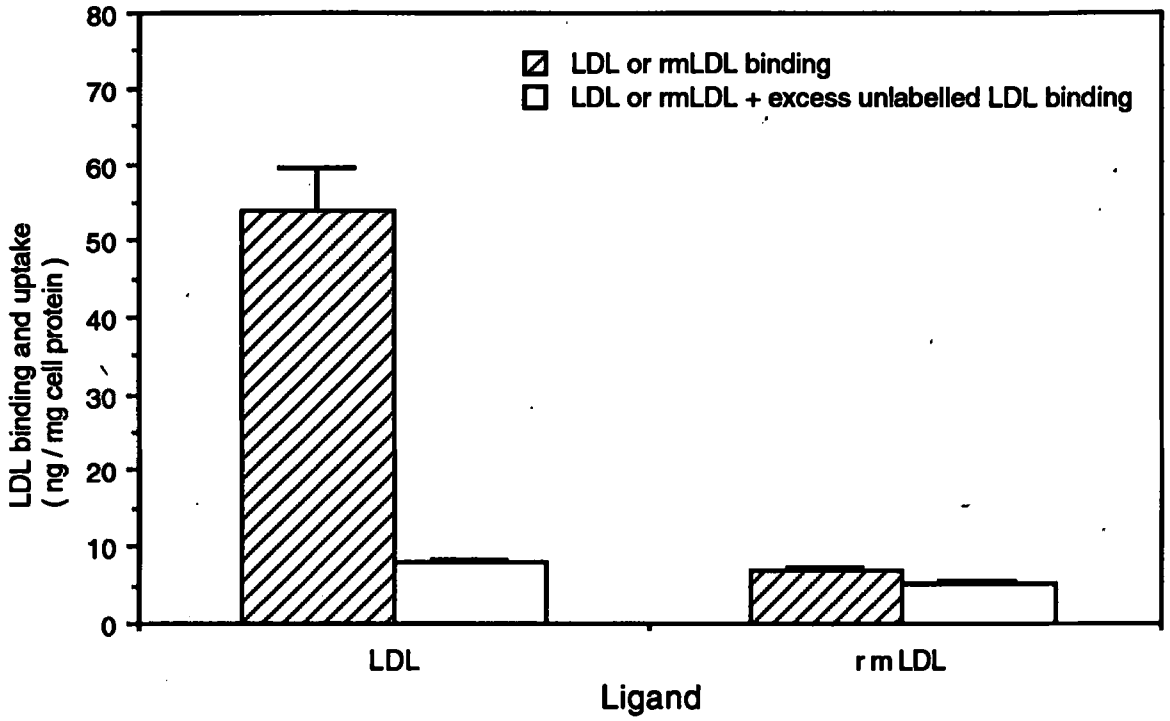


Figure 5.2: Measurement of LDL receptor activity on HepG2 cells in dishes; LDL versus rmLDL

HepG2 cells were plated out at a density of 150,000 cells per 1.77cm^2 dish, in 1ml of R10+Glu. On day one they were re-fed with R10+Glu. On day four, the cells were pre-washed with 1% defatted BSA, twenty minutes per wash then assayed at 37°C in the usual way (see 2.1.3 and 2.1.5), but using ^{125}I-LDL to measure total binding, ^{125}I-rmLDL +/- unlabelled LDL and ^{125}I-LDL+excess unlabelled LDL to measure non-specific binding (see below).

1µg/ml ^{125}I-LDL	(total binding)
1µg/ml ^{125}I-LDL + 250µg/ml unlabelled LDL	(non-specific binding)
1µg/ml ^{125}I-rmLDL	(total binding)
1µg/ml ^{125}I-rmLDL + 250µg/ml unlabelled LDL	(non-specific binding)

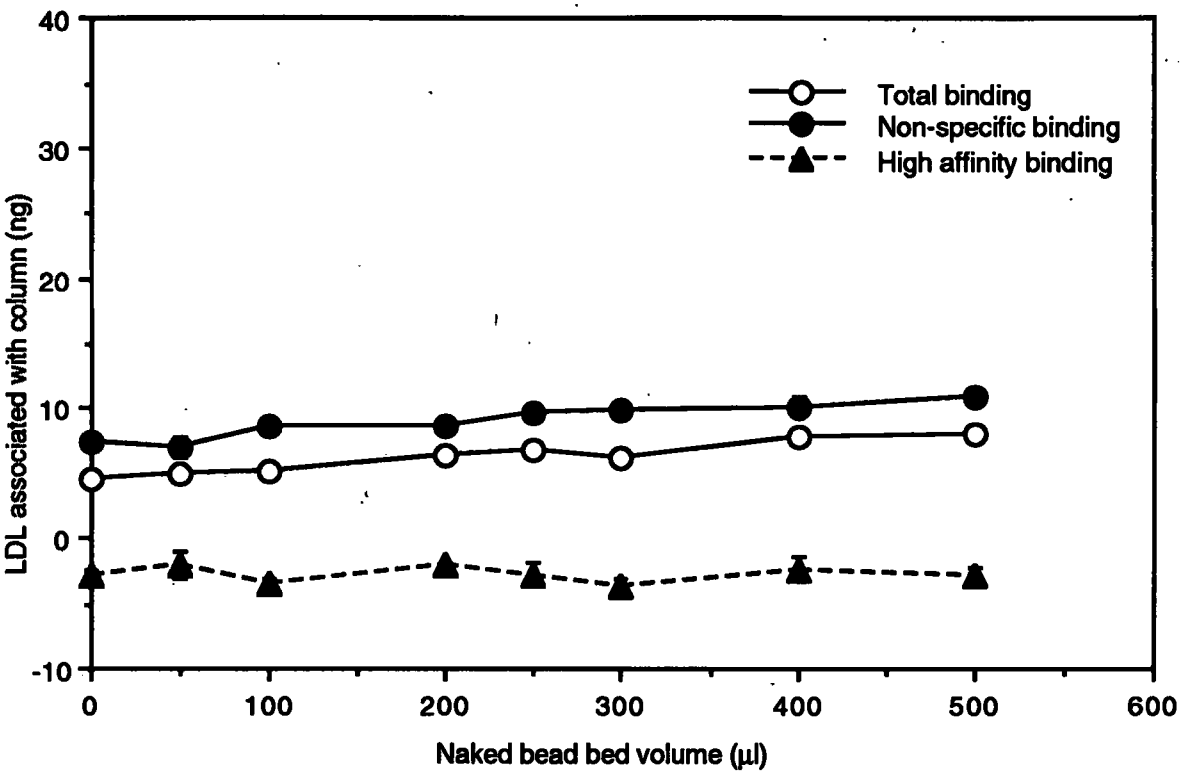


Figure 5.3 Binding activity of ^{125}I-LDL, and ^{125}I-reductively methylated LDL to naked Cytodex 2 beads in columns at 37°C.

Increasing volumes of naked Cytodex 2 beads were poured into columns and assayed using 1µg/ml ^{125}I-LDL to measure total binding, and 1µg/ml ^{125}I-reductively methylated LDL to measure non-specific binding. High affinity was found by subtracting non-specific binding from total binding.

5.4.2. LDL versus reductively methylated LDL, receptor activity on HepG2 cells in dishes and on beads.

5.4.2a. ^{125}I-LDL and ^{125}I-reductively methylated LDL binding to naked beads.

The second method was to use ^{125}I-LDL to measure total binding and uptake, and ^{125}I-reductively methylated LDL (^{125}I-rmLDL) to measure non-specific binding and uptake. Weisgraber et al in 1978 showed that the reductive methylation of LDL (method Chapter 2), only modified the amino acid lysine, and that the major product formed was dimethyllysine. This process did not change the net charge of the lipoprotein, but with methylation of approximately 9.7 lysine residues out of 20 achieved in abolishing LDL binding activity. The method used for reductive methylation for these assays involved the reductive methylation of 13 lysine groups, therefore abolishing binding activity, but keeping the overall positive charge the same [Weisgraber K. H. et al 1978].

In the experiment shown in Figure 5.2 HepG2 cells were grown in dishes and assayed in the standard way using 1μg/ml ^{125}I-LDL to measure total binding, and 1μg/ml ^{125}I-LDL+250μg/ml unlabelled LDL, 1μg/ml ^{125}I-rmLDL, and 1μg/ml ^{125}I-rmLDL+250μg/ml unlabelled LDL to measure non-specific binding. From this graph it can be seen that ^{125}I-reductively methylated LDL, either in the presence or absence of excess unlabelled LDL, produced the same values for non-specific binding and uptake, as that obtained by using ^{125}I-LDL+excess unlabelled LDL. This demonstrates that ^{125}I-rmLDL can be used to determine non-specific binding, and that LDL does not compete with rmLDL for non-specific binding sites. The small amount of rmLDL required meant that the demand for blood donors, for the fresh supply of human LDL was greatly reduced.

In Figure 5.3 (Binding activity of ^{125}I-LDL and ^{125}I-reductively methylated LDL to naked Cytodex 2 beads in columns at 37°C), increasing volumes of naked Cytodex 2 beads were poured into columns and assayed using 1μg/ml ^{125}I-LDL to measure total binding, and 1μg/ml ^{125}I-reductively methylated LDL to measure non-specific binding.

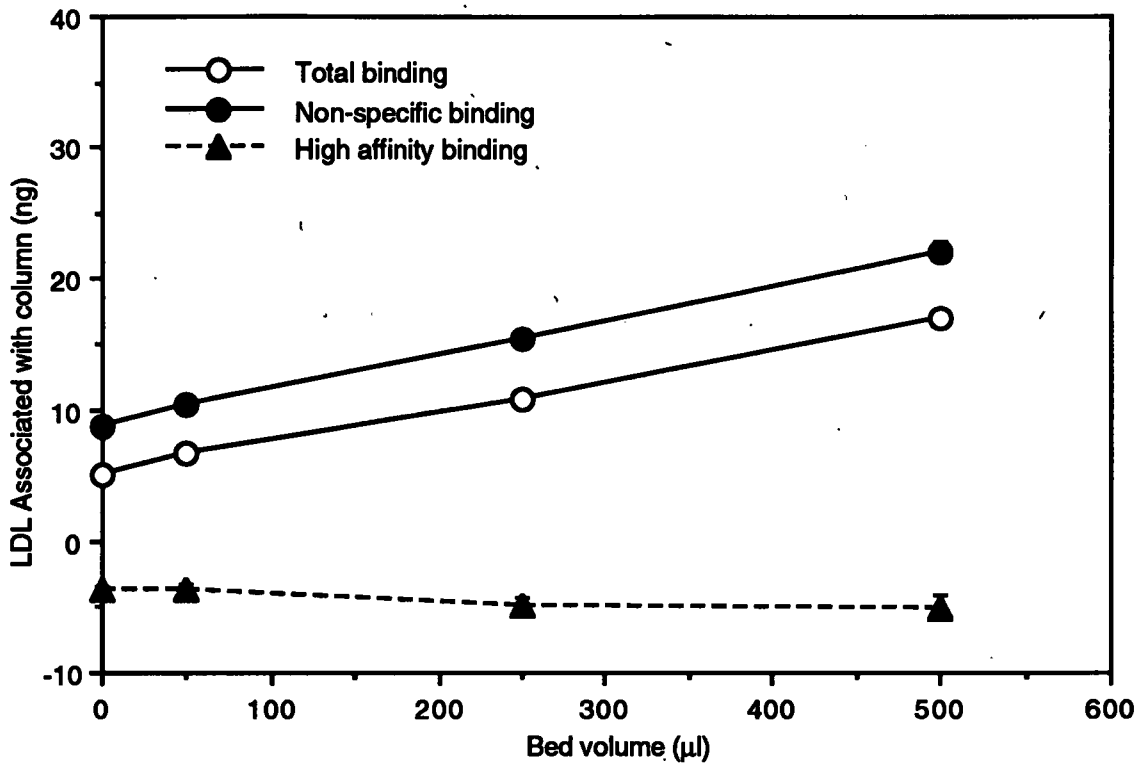


Figure 5.4(a). Various volumes of HepG2 cells on beads grown in BLPDS+25OHch.

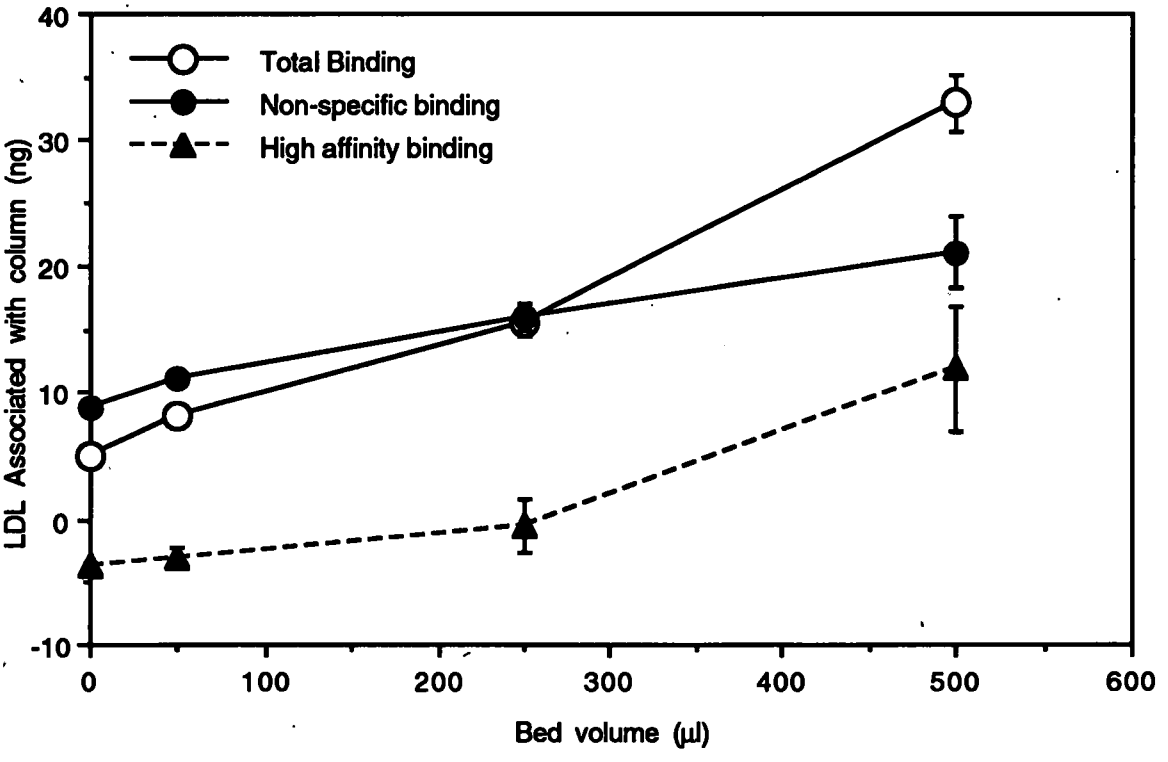


Figure 5.4(b) Various volumes of HepG2 cells on beads grown in BLPDS-25OHch.

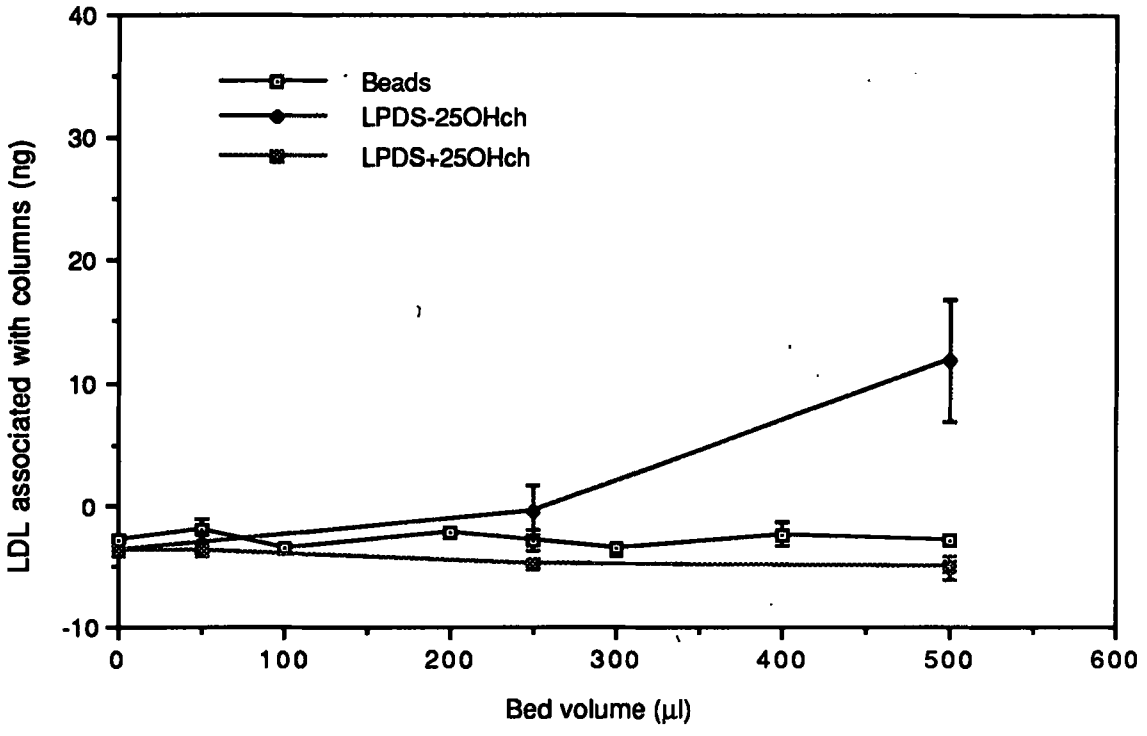


Figure 5.4(c). High affinity (or receptor mediated) binding of HepG2 cells on beads in BLPDS in the presence or absence of 25 hydroxycholesterol, compared with naked beads (Figure 5.3).

Figure 5.4(a,b,c,) Binding and uptake of ^{125}I-LDL, and ^{125}I-reductively methylated LDL to HepG2 cells cultured on Cytodex 2 beads in the presence and absence of 25 hydroxycholesterol.

Two stirrer bottles were employed for this experiment. The standard plating and cell attachment protocol was utilised for the initial 24 hours. One stirrer bottle was then continued containing 10% BLPDS, whilst in the second stirrer bottle the medium was adjusted to 10% BLPDS + 2.5µM 25 hydroxycholesterol.

Increasing volumes of HepG2 cells on beads were poured into columns and assayed using 1µg/ml ^{125}I-LDL to measure total binding, and 1µg/ml ^{125}I-rmLDL to measure non-specific binding. High affinity (or specific binding) was obtained by subtracting non-specific from total binding.

High affinity binding was then calculated by subtracting non-specific binding from total binding. These results show that a) ^{125}I-rmLDL bound more readily to the columns than naked beads, and b) neither rmLDL nor LDL bound to the naked Cytodex 2 beads (increasing numbers of naked beads did not increase ^{125}I-ligand binding).

5.4.2b. Using ^{125}I-LDL to measure modulation of the LDL receptor on HepG2 cells on beads.

These experiments were carried out using HepG2 cells which had been grown on Cytodex 2 beads in stirrer bottles in 10%BLPDS in the presence or absence of 25 hydroxycholesterol. This was to establish if the LDL receptor on the HepG2 cells could be down regulated (25 hydroxycholesterol reduces the LDL promoter gene activity [Takagi, et al ., 1988]). This was carried out by pouring increasing volumes of cells on beads into columns, then performing a 37°C assay using 1μg/ml ^{125}I-LDL to measure total binding, and ^{125}I-rmLDL to measure non-specific binding. High affinity (receptor-mediated, or specific) binding was calculated by subtracting non-specific from total binding.

In the presence of 25 hydroxycholesterol, when receptor activity would be expected to be low or absent, increased volumes of HepG2 cells on Cytodex 2 beads gave increased values of both ^{125}I-LDL, and ^{125}I-rmLDL (Figure 5.4a). This meant that both ligands bound increasingly with increased cell numbers, and not simply with increased bead volume (see Figure 5.3).

The data illustrated in Figure 5.4b), (Various volumes of HepG2 cells + beads grown in BLPDS - 25 hydroxycholesterol) showed that in the absence of 25 hydroxycholesterol, receptor activity could be measured. However, due to the fact that both rmLDL and LDL bound to the column material, and were dependent on cell number, it is difficult to know what non-specific binding was actually present, and therefore ultimately what receptor-mediated activity was present.

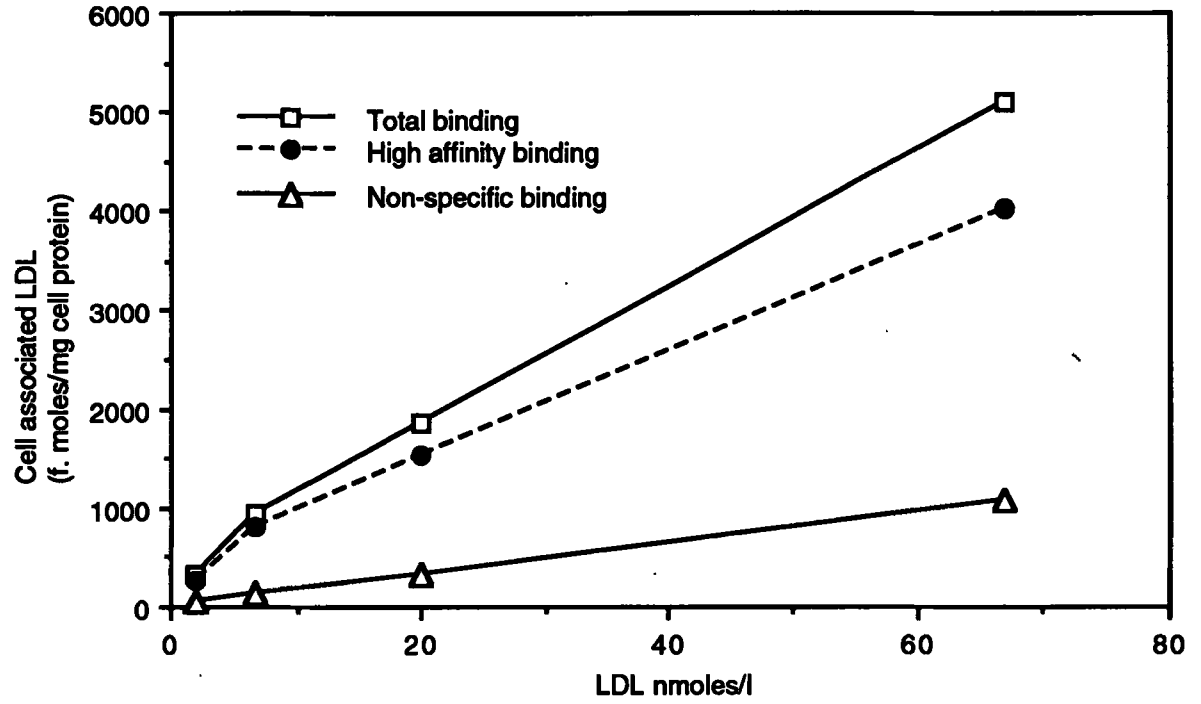
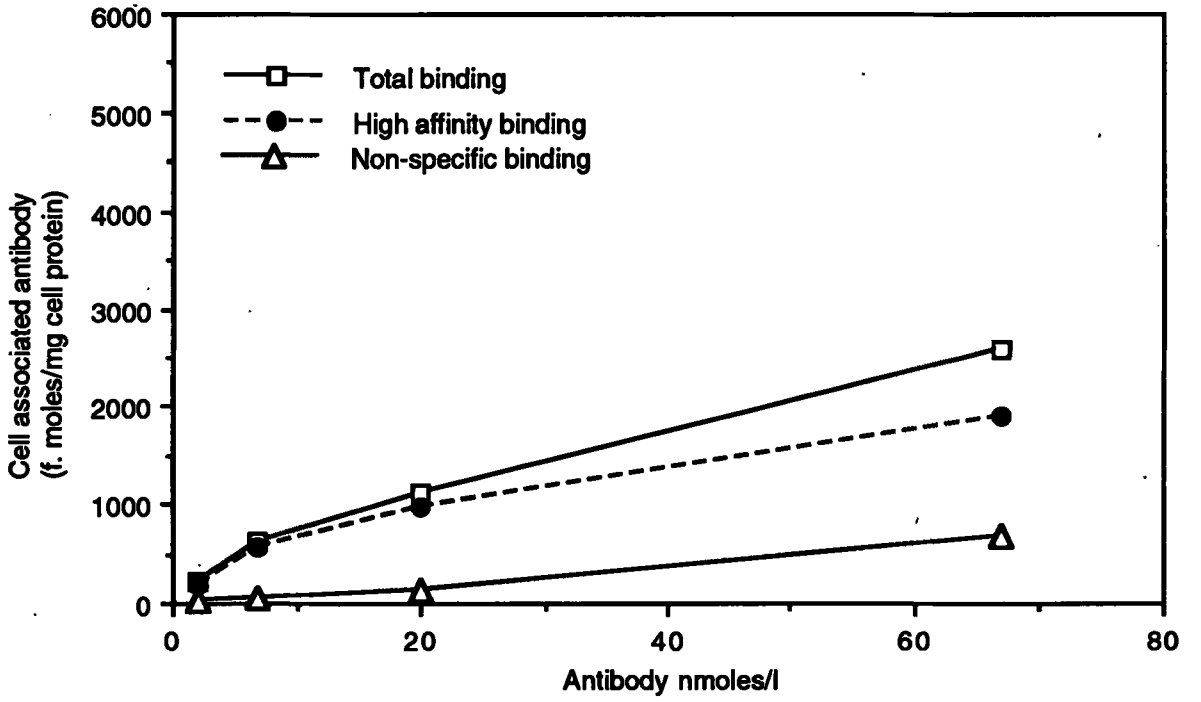


Figure 5.5(a) Binding and uptake of LDL.



Figures 5.5 (b) Binding and uptake of C7.

HepG2 cells were plated out at a density of 150,000 per well in 1ml of R10+Glu. On day two they were washed with PBS and re-fed with 10% BLPDS. On day three an ^{125}I-ligand 37°C assay was performed using concentrations of LDL (a) or antibody (b).

When the results from Figure 5.4a. 5.4b. and 5.3 were plotted on the same graph, Figure 5.4c, (Binding and uptake of ^{125}I-LDL, and ^{125}I-reductively methylated LDL to HepG2 cells cultured on Cytodex 2 beads in the presence and absence of 25 hydroxycholesterol) the problem of interpreting results using the ^{125}I-LDL and ^{125}I-rmLDL was emphasised, in that the LDL receptor-mediated activity observed after the cells had been incubated with 25 hydroxycholesterol was lower than that associated with the column and naked Cytodex 2 beads.

5.4.3. LDL versus Antibody, for the measurement of LDL receptor-mediated binding and uptake at 37°C on HepG2 cells grown in dishes.

The third and final method was to measure the LDL receptor on HepG2 cells in columns by using ^{125}I-C7 and ^{125}I-Myb$^{2-3}$ monoclonal antibodies. C7 is a monoclonal antibody, directed against the LDL receptor, which binds to the first cysteine rich repeat in the ligand-binding domain (2-42 amino acids) of the receptor and was used to measure total binding (described in 5.1.4).

5.4.3a. LDL versus Antibody for the measurement of LDL receptor activity of HepG2 cells grown in dishes.

Initially it was important to ascertain the suitability of the ^{125}I-antibody, in the measurement of the LDL receptor on the HepG2 cells grown in tissue culture dishes, compared with that of ^{125}I-LDL. This was achieved by following some of the methods carried out by Beisiegel in 1981 [Beisiegel et al., 1981] on fibroblasts, but using HepG2 cells.

HepG2 cells in this set of experiments were plated out in dishes in R10+Glu, then re-fed on day two with BLPDS. On day three an ^{125}I-Ligand assay was performed at 37°C using increasing concentrations of LDL (a) or antibody (b).

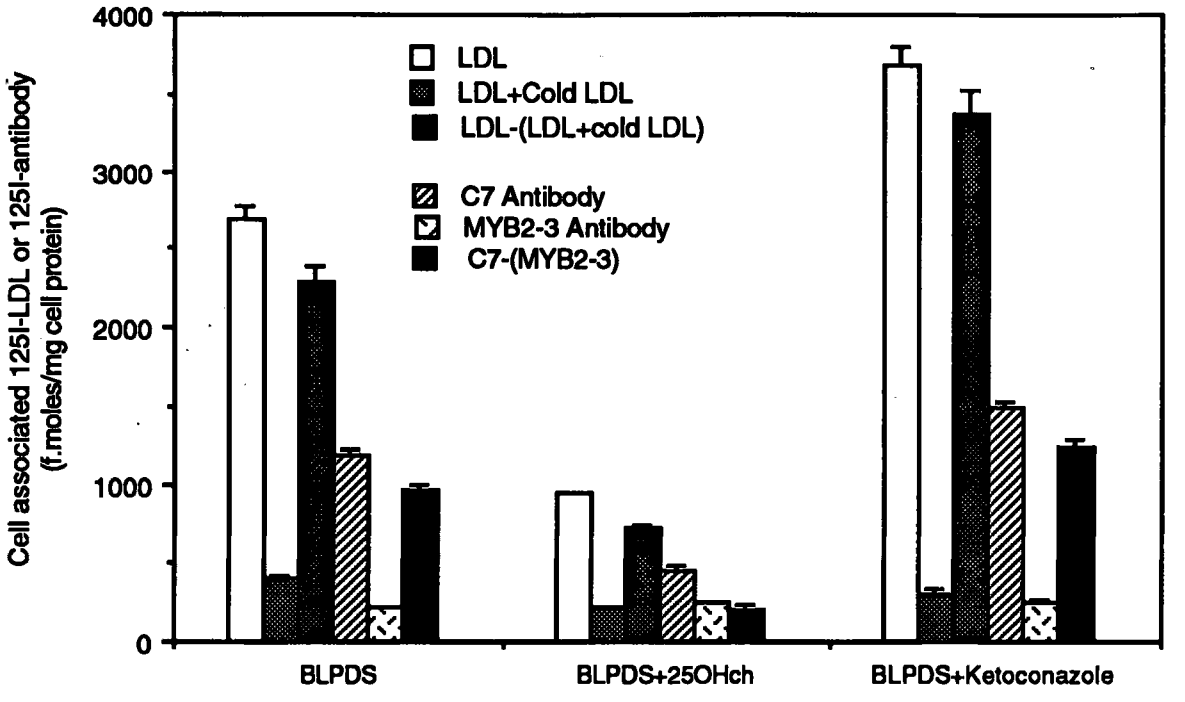


Figure 5.6 HepG2 cells grown on tissue culture plastic in 10%BLPDS in the presence or absence of 2.5μM 25 hydroxycholesterol, and in the presence or absence of 30μM Ketoconazole.

HepG2 cells were plated out at a density of 300,000 cells per 1.77cm^2 dish, in 1ml of R10+Glu. day one they were washed with PBS and re-fed with 10%BLPDS in the presence or absence of 2.5 μM 25 hydroxycholesterol, and in the presence or absence of 30 μM Ketoconazole. On day two a ^{125}I-Ligand 37°C internalisation assay was performed using either 3μg/ml ^{125}I-LDL, plus ^{125}I-LDL with excess LDL (250 μg/ml), or ^{125}I-C7 and 3μg/ml ^{125}I-Myb$^{2-3}$.

Figure 5.5 a. (Binding and uptake of LDL) shows the results of binding and uptake expriments where ^{125}I-LDL was used to measure total binding and ^{125}I-LDL plus excess unlabelled LDL was used to measure non-specific binding. High affinity binding was calculated by subtracting non-specific binding from total binding. These can be compared to results shown in Figure 5.5.b. (Binding and uptake of C7) measured by ^{125}I-C7 (total binding), and ^{125}I-Myb$^{2-3}$ (non-specific binding). High affinity is calculated as before by subtracting non-specific values from total binding values. It can be seen, that at all concentrations measured, the amount of C7 bound and taken up is approximately half that of LDL (C7 binds to a maximum of 50-70% of LDL and βVLDL binding sites [Beisiegal et al ., 1981]).

5.4.3b. Measurement of up- and down-regulation of HepG2 cells grown in dishes using ^{125}I-Antibodies (C7 and Myb$^{2-3}$).

The second approach was to see if up- and down-regulation of the LDL receptor on HepG2 cells, in tissue culture dishes, could be measured by using ^{125}I-C7, and whether ^{125}I-Myb$^{2-3}$ was suitable for measuring non-specific binding.

In Figure 5.6 HepG2 cells grown on tissue dishes in 10% BLPDS in the presence or absence of 2.5μM 25 hydroxycholesterol, and in the presence or absence of 30μM ketoconazole. The graph shows that non-specific binding and uptake was constant in all cases using Myb$^{2-3}$, proving it was a suitable antibody in the measurement of non-specific binding. This experiment also showed maximal up-regulation, in the presence of Ketoconazole (which inhibits P450 enzymes involved in cholesterol synthesis [Feldman D. et al ., 1986]), and maximal down-regulation by 25 hydroxycholesterol (which reduces the LDL receptor genes promoter activity [Takagi et al ., 1989]).

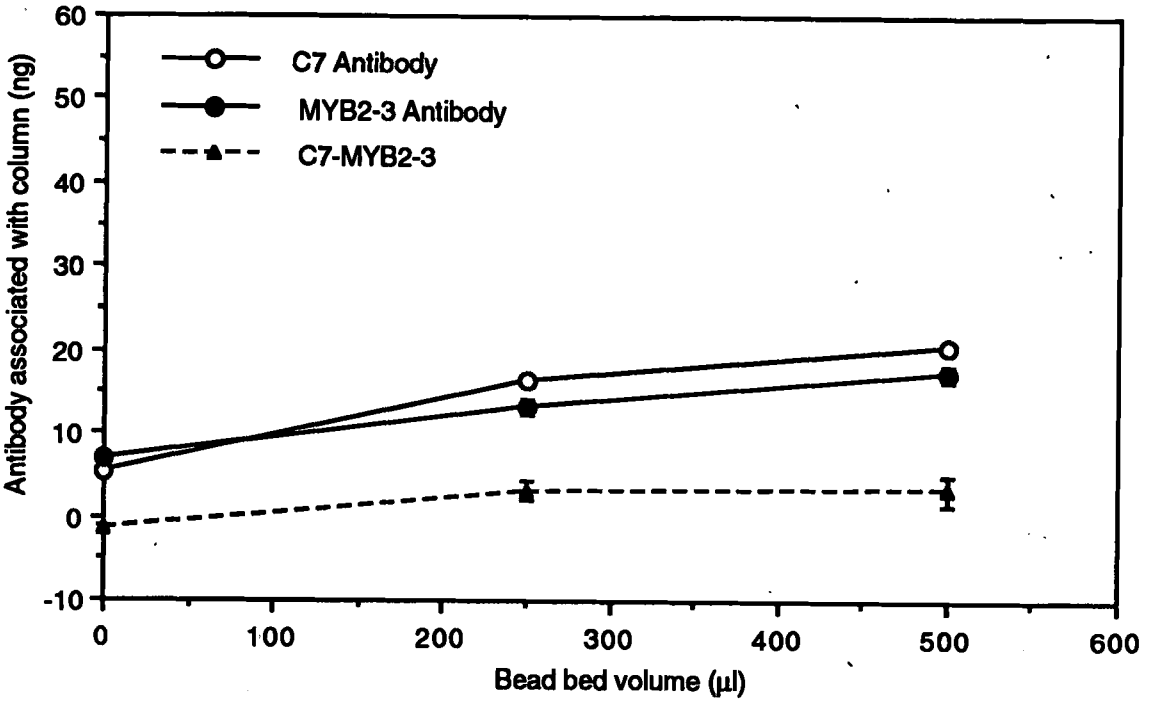


Figure 5.7 Binding activity of ^{125}I-C7, and ^{125}I-Myb$^{2-3}$ to naked Cytodex 2 beads. Increasing volumes of naked Cytodex 2 beads were poured into columns and assayed using 3µg/ml ^{125}I-C7 to measure total binding, and 3µg/ml ^{125}I-Myb$^{2-3}$ to measure non-specific binding. High affinity was found by subtracting non-specific binding from total binding.

5.4.4. Suitability of ^{125}I-Antibody for the measurement of the LDL receptor on HepG2 cells grown on beads.

The third and final approach in this section was to repeat the methods used when testing ^{125}I-LDL and ^{125}I-rmLDL, with the antibodies C7 and Myb$^{2-3}$.

5.4.4a. ^{125}I-C7 and ^{125}I-Myb$^{2-3}$ binding to naked beads.

In the first experiment increased volumes of naked Cytodex 2 beads were poured into columns, this was to establish the amount of ^{125}I-C7 and ^{125}I-Myb$^{2-3}$ which would bind to the column and the Cytodex 2 beads. Figure 5.7 (Binding activity of ^{125}I-C7, and ^{125}I-Myb$^{2-3}$ to Cytodex 2 beads), shows that between 250µl and 500µl volumes of naked Cytodex 2 beads, no increase in pseudo high affinity binding was observed (i.e. C7:Myb$^{2-3}$ binding values, or non-specific subtracted from total binding).

5.4.4b. Using ^{125}I-LDL and ^{125}I-rmLDL to measure modulation of the LDL receptor on HepG2 cells on beads.

The second experiment involved growing HepG2 cells on Cytodex 2 beads in the presence or absence of 25 hydroxycholesterol. Increased amounts of these beads with confluent HepG2 cells were poured into columns and assayed using 3µg/ml ^{125}I-C7 to measure total binding and uptake, 3µg/ml ^{125}I-Myb$^{2-3}$ to measure non-specific binding and uptake. High affinity was calculated by subtracting non-specific from total values. The results of which are as follows:-

Figure 5.8a. (Various volumes of HepG2 cells on beads grown in BLPDS-25OHch (25 hydroxycholesterol)), shows that in the absence of 25 hydroxycholesterol an increase in total binding and uptake (^{125}I-C7), and therefore high affinity or specific binding and uptake (C7-Myb$^{2-3}$), with no obvious increase in non-specific binding (^{125}I-Myb$^{2-3}$).

Figure 5.8b. (Various volumes of HepG2 cells on beads grown in BLPDS+25 OHch), shows that HepG2 cells grown in the presence of 10% BLPDS, and 25

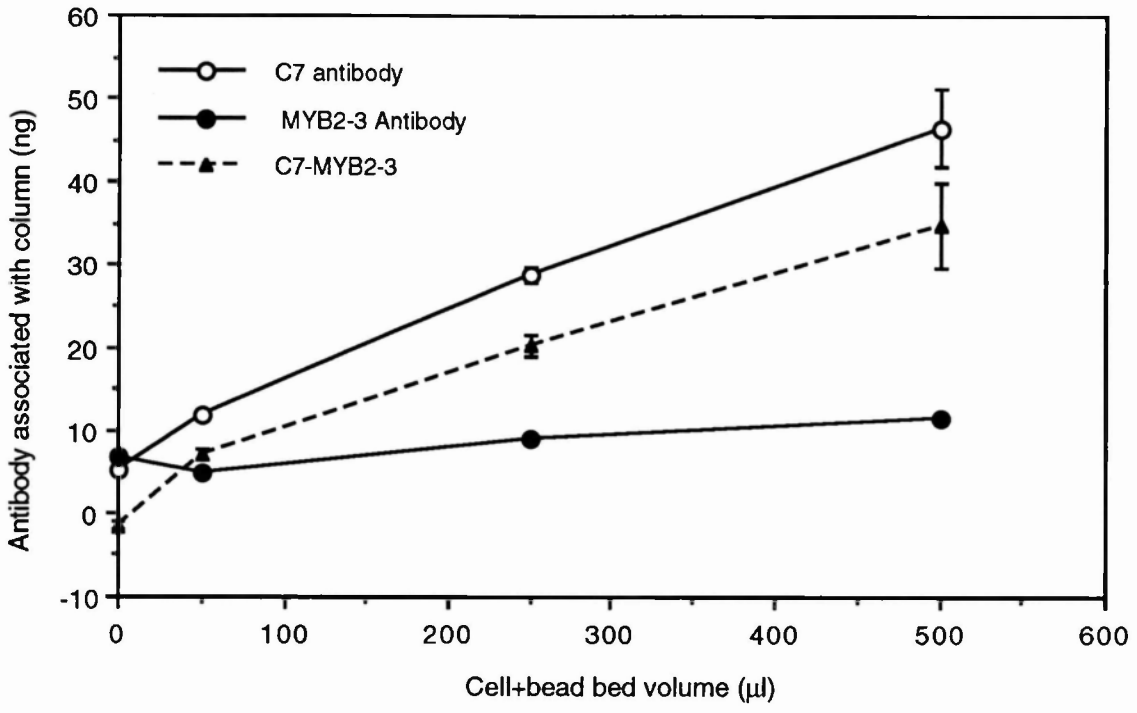


Figure 5.8(a). Various volumes of HepG2 cells on beads grown in BLPDS-25OHch.

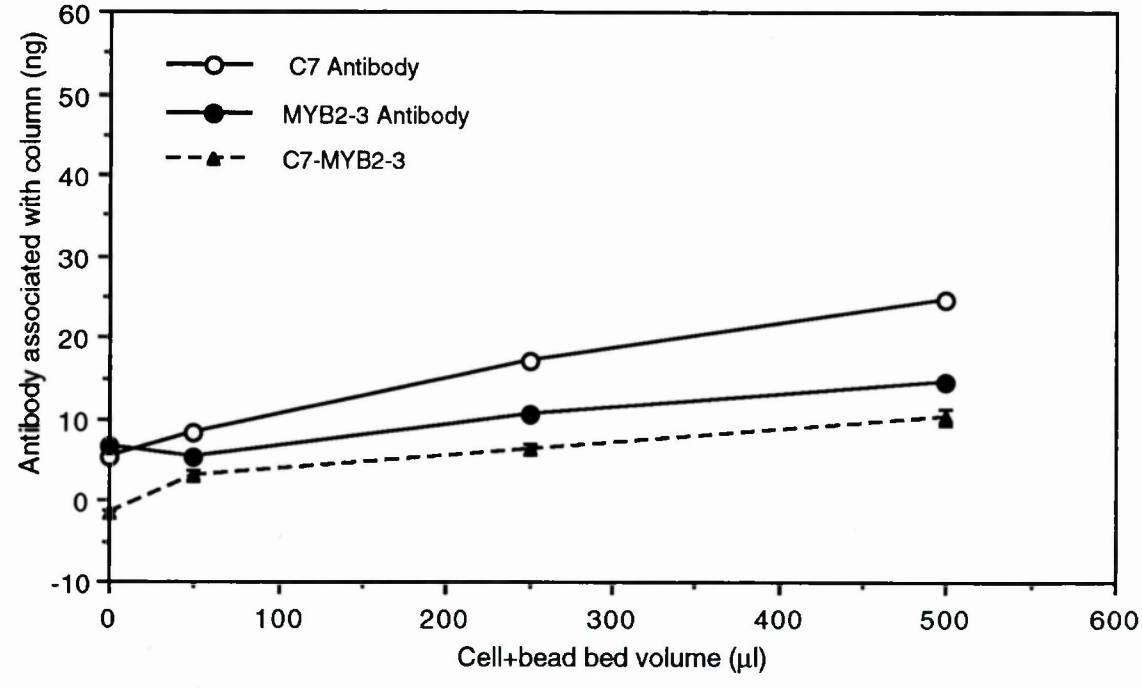


Figure 5.8 (b). Various volumes of HepG2 cells on beads grown in BLPDS+25OHch

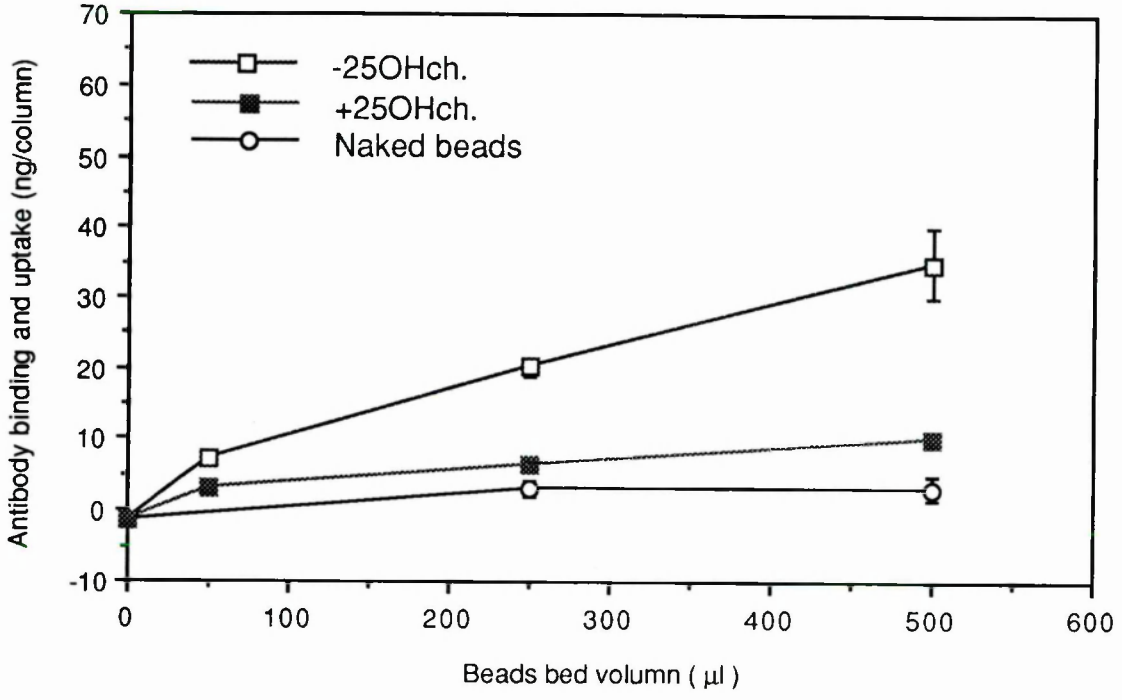


Figure 5.8.(c). High affinity (or receptor mediated) binding of HepG2 cells on Cytodex 2 beads in BLPDS in the presence or absence of 25 hydroxycholesterol, compared with naked beads.

Figure 5.8.(a,b,c,). Binding and uptake of ^{125}I-C7, and ^{125}I-Myb$^{2-3}$ to HepG2 cells cultured on Cytodex 2 beads in the presence or absence of 25 hydroxycholesterol.

Two stirrer bottles were employed for this experiment. The standard plating and cell attachment protocol was utilised for the initial 24 hours. Incubation of one stirrer bottle was then continued with medium containing 10%BLPDS, whilst the incubation of the second was continued with 10% BLPDS + 2.5µM 25 hydroxycholesterol.

Increased volumes of HepG2 cells on beads into columns was assayed using 3µg/ml ^{125}I-C7 to measure total binding, and ^{125}I-Myb$^{2-3}$ to measure non- specific binding. High affinity (or receptor mediated) binding was obtained by subtracting non-specific from total binding.

hydroxycholesterol in stirrer bottles, then poured into columns and assayed, showed little total binding (^{125}I-C7), and non-specific binding (^{125}I-Myb$^{2-3}$), as the volume of cells on beads increased.

Finally high affinity (specific, or receptor mediated) binding, of HepG2 cells on Cytodex 2 beads in the presence or absence of 25 hydroxycholesterol, was compared with binding on naked Cytodex 2 beads. The results are shown in Figure 5.8c, which demonstrates that pseudo high affinity binding of naked beads is approximately zero, and that HepG2 cells grown in the presence of 10%BLPDS, and in the absence of 25 hydroxycholesterol are up-regulated. HepG2 cells grown in the presence of both 10%BLPDS and 25 hydroxycholesterol are fully down-regulated.

Chapter 6

PERFUSION COLUMN SYSTEM

6.1. Introduction.

Hepatocytes control plasma cholesterol concentration by both secreting and removing cholesterol-carrying lipoproteins from the blood. Thus hepatocytes release very low density lipoprotein (VLDL) into the blood where it is converted to low density lipoprotein (LDL). LDL is eventually removed, mainly via LDL receptors on hepatocytes (discussed in Chapter 1 also see Figure 1.4.).

A common technique for investigation of the LDL receptor expression is the use of the human hepatoma cell line, HepG2, grown in monolayer culture. In this system, however, cells are in prolonged contact with medium containing the cell's own secretions, e.g. lipoproteins and bile acids, which may perturb cellular metabolism and alter LDL receptor expression. As discussed in Chapter 1, the processes of lipoprotein secretion and uptake are separated in the liver, in that they are not bathed in their own secreted material [Reid et al., 1992]. This problem would be overcome in a perfused system in which cell products are removed by continuously renewing the culture medium.

In the past, various studies have been carried out on perfused cells. These were usually grown on supporting beads such as Cytodex. The majority of these studies have been on endothelial cells, so that the secretions and chemical interactions could be studied [Busch et al., 1982], (this will be discussed more fully in Chapter 8). To my knowledge normal human liver cells (hepatocytes), have not been perfused in this way, Visvikis [Visvikis et al., 1990] however did grow HepG2 cells on Cytodex 3 beads (mentioned in Chapter 3 regarding seeding density), in stirrer bottles, so that the production of gamma glutamyltransferase could be analysed and measured. In addition, a rat hepatocyte cell line H-4-11-E-C3 has been grown on Cytodex 3 beads and perfused, enabling the study of insulin inhibiting PEPCK mRNA levels stimulated by cAMP and dexamethasone [Courtney Harrison Jr. H. et al., 1991].

6.2. Aim.

The aim of these experiment were to:-

1. Feed the HepG2 cells in columns by both single pass and reperfusion over increasing periods of time in order to observe the effects, if any, of this treatment on protein concentration, morphological appearance and alteration in LDL receptor binding and uptake.
2. Observe the effect of conditioned medium produced by HepG2 cells in the presence of BLPDS, and in the presence of BLPDS plus varying concentrations of glucose, on the LDL receptor.
3. Produce maximal and minimal regulation of the LDL receptor by single pass perfusion with 2.5μM 25 hydroxycholesterol or 30μM Ketoconazole.
4. Make a comparison of single pass versus reperfusion using 25 hydroxycholesterol.

6.3. Methods.

All standard methods used in the experiments in this Chapter have been described in detail in Chapter 4., and Chapter 5. namely:-

Plating and cell attachment.

Pouring columns.

LDL receptor assay and/or perfusion.

Measurement of ^{125}I-ligand (antibody) binding and uptake at 37°C in columns and in dishes.

Radiolabelling of ligands.

Quality control of ^{125}I-labelled antibodies.

Calculation of standard error of the mean (s.e.m.), which are shown as error bars on the graphs.

Any variation to this will be noted with the appropriate graph.

Statistics:- In addition to the s.e.m. values quoted in previous Chapters, statistical analysis for significant differences between means was carried out using Student's t-test. This was done using the Microsoft Excel version 4 package, t-test: two sample assuming unequal variances. Interpretation of results were as follows, $P< 0.05$ is significant, $P< 0.01$ is very significant, $P< 0.001$ is highly significant.

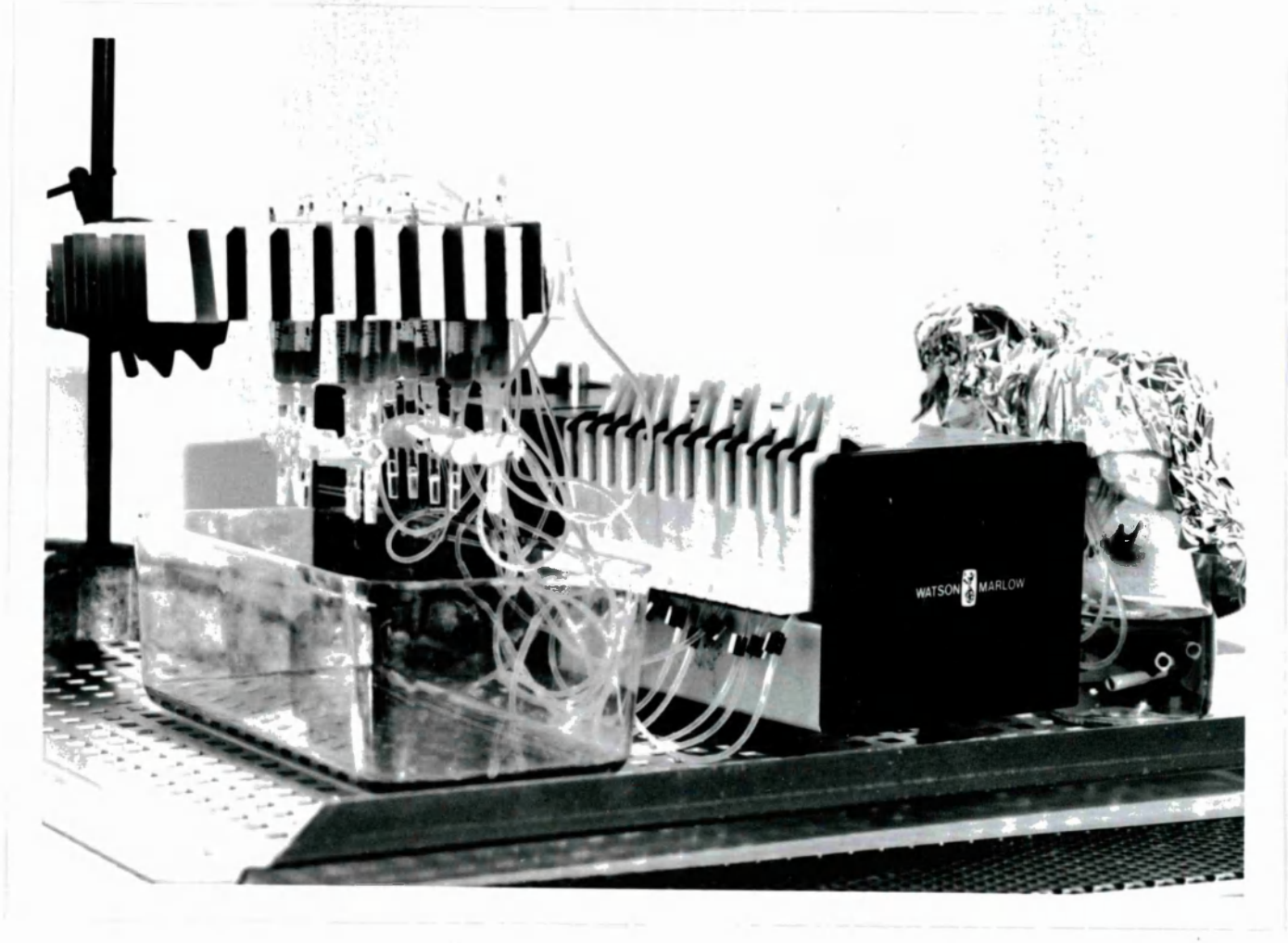


Plate 10: Photograph of Single pass perfusion column system.

6.4. Results and Discussion.

Perfusion is the process in which the cells on beads in columns are gently bathed in medium from which they receive nutrients. This process has been achieved in this system by either single pass perfusion, which is an open system that enables medium to flow through the column so that cells constantly receive fresh medium, or, by reperfusion, that is a closed system whereby medium is continuously recycled.

Plate 10 shows a photograph of the single pass perfusion system. Both systems are diagrammatically represented in Figure 4.2. (Perfusion culture of HepG2 cells on Cytodex 2 microcarrier beads).

6.4a. To determine whether HepG2 cells could be perfused over a twenty-four period in the column system.

When the system was first designed it was important to establish that receptor activity could be measured over a set period, the period decided upon was eighteen to twenty-four hours. This proved to be sufficient to enable any harmful effects that the system may have on the cells, to become apparent. Also, if the cells were to be subjected to compounds which may effect the LDL receptor such as 2.5μM 25 hydroxycholesterol (which down regulates the receptor), or 30μM Ketoconazole (which up regulates the receptor), this time period would then be sufficient to observe any effects produced by this treatment.

The first experiment carried out was to determine if high affinity binding could be measured in the column system after perfusion. It was carried out by pouring HepG2 cells into columns, subjecting them to reperfusion for time intervals of zero, two, six, and eighteen hours with 3mls of 10% BLPDS, at a speed of 1ml per hour. LDL receptor activity was then measured in the normal way at 37°C, using 3μg/ml ^{125}I-C7 to measure total binding, and ^{125}I-Myb$^{2-3}$ to measure non-specific binding. High affinity binding was calculated by subtracting non-specific from total binding values.

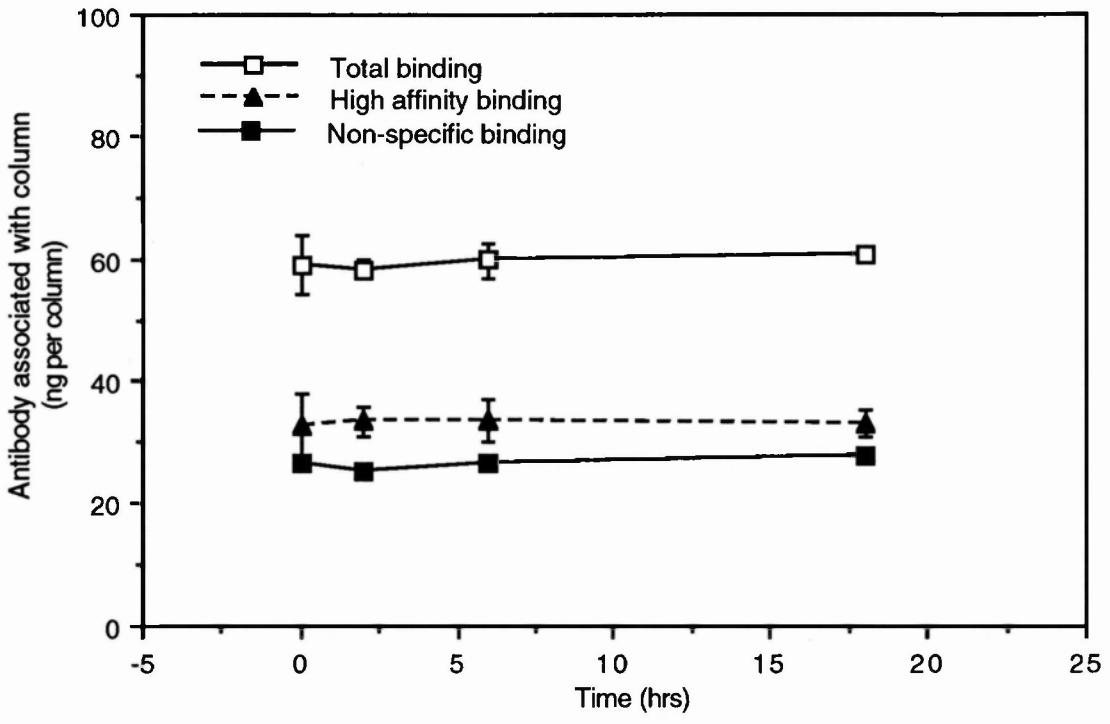


Figure 6.1. Binding and uptake at 37°C by the LDL receptor, on HepG2 cells reperfused in columns over increasing time intervals.

Standard method for plating, attachment and pouring of 500µl cell/beads columns. 3mls of 10%BLPDS was reperfused in six columns for each time point. Three columns to measure total binding, and three columns to measure non-specific binding. High affinity or receptor - mediated binding was calculated by subtracting non-specific from total binding.

The pump used had only 12 channels, therefore the experiment had to be carried out on two consecutive days. That is, columns were poured for time intervals zero, two and six hours from one set of stirrer bottles, then the following day columns were poured for time intervals zero and eighteen hours. Time point zero total, non-specific and high affinity results were calculated by combining both sets of zero results.

Plate assays were performed at time point zero, six and eighteen hours to validate the antibodies. Data not shown.

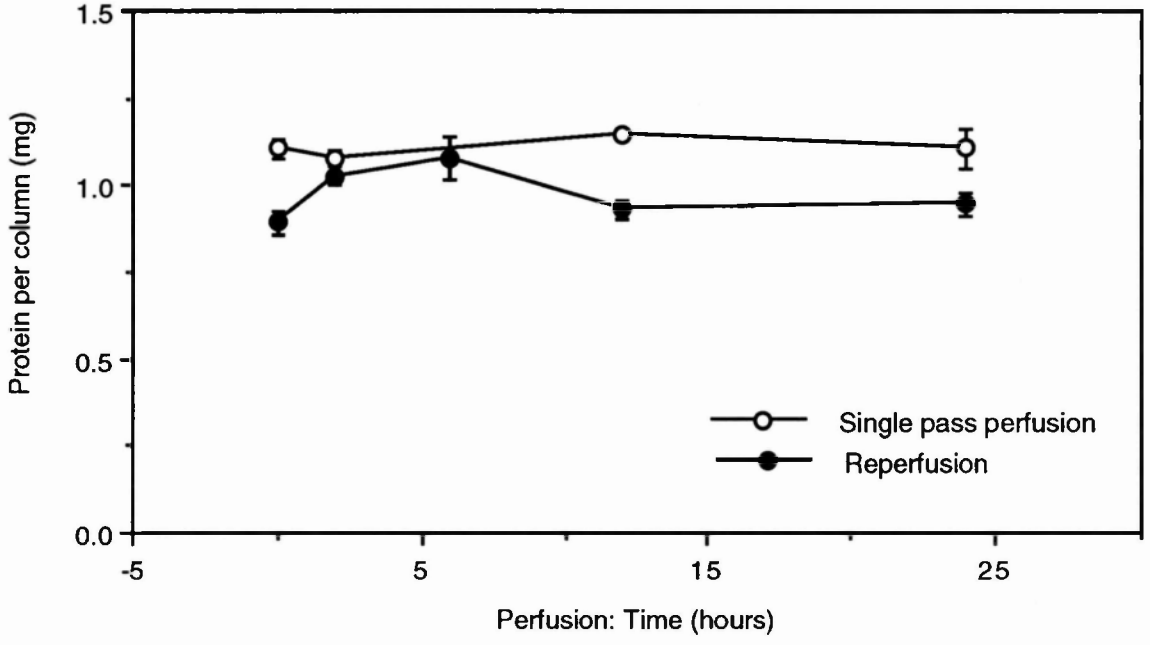


Figure 6.2. Protein concentrations measured in columns. Single pass versus reperfusion over increasing time intervals 0-24 hours.

Standard method for plating, attachment and pouring of 500µl cell/beads columns 3mls. of 10%BLPDS was used in the perfusion columns, and a continuous fresh supply of 10% BLPDS was used for the single pass perfusion columns. Protein assays were carried out using the Lowry method. The protein and s.e.m. values for each time point was calculated from three columns per condition.

Figure 6.1. (Binding and uptake at 37°C by the LDL receptor, on HepG2 cells reperfused in columns over increasing time intervals) shows that over the eighteen hour period of reperfusion of 3mls of 10%BLPDS the LDL receptor binding and uptake at 37°C remained constant.

Once it had been established that high affinity binding and uptake at 37°C could be measured on HepG2 cells which had been reperfused with medium for up to eighteen hours, it was important to find if perfusion was damaging the cells. This was investigated in two ways, firstly by carrying out protein assays and secondly by scanning electron microscopy (S.E.M.).

Protein: The protein assays were carried out using the Lowry method as described in Chapter 2. at set time intervals over a twenty-four hour period, on cells in columns which had either been a) reperfused with 3mls of 10%BLPDS or b) single pass perfused with fresh 10%BLPDS. The results of these can be seen in Figure 6.2. (Protein concentrations measured in columns. Single pass versus reperfusion over increasing time intervals 0-24 hours). It can be seen that with single pass perfusion there was no significant change in protein per column over twenty-four hours. The overall protein was 1.11mg/column (s.e.m. +/- 0.023). For cells perfused by reperfusion the protein concentration appeared to vary slightly over twenty-four hours increasing slightly at two and six hours, but being the same as at zero time by twelve and twenty-four hours. The overall mean protein was 0.98mg/column (s.e.m. +/- 0.010). Clearly the mean value of protein/column was slightly lower initially in the columns used for the reperfusion experiments (0.81 +/- 0.020 mg/column), than in the columns used for single pass perfusion (1.21 +/- 0.019 mg/column).

Scanning electron microscopy: HepG2 cells on Cytodex 2 beads were fed by single pass perfusion or reperfusion, then at set time intervals of zero, two, six and twenty-four hours they were prepared for electron microscopy. The fixation for this was carried out using

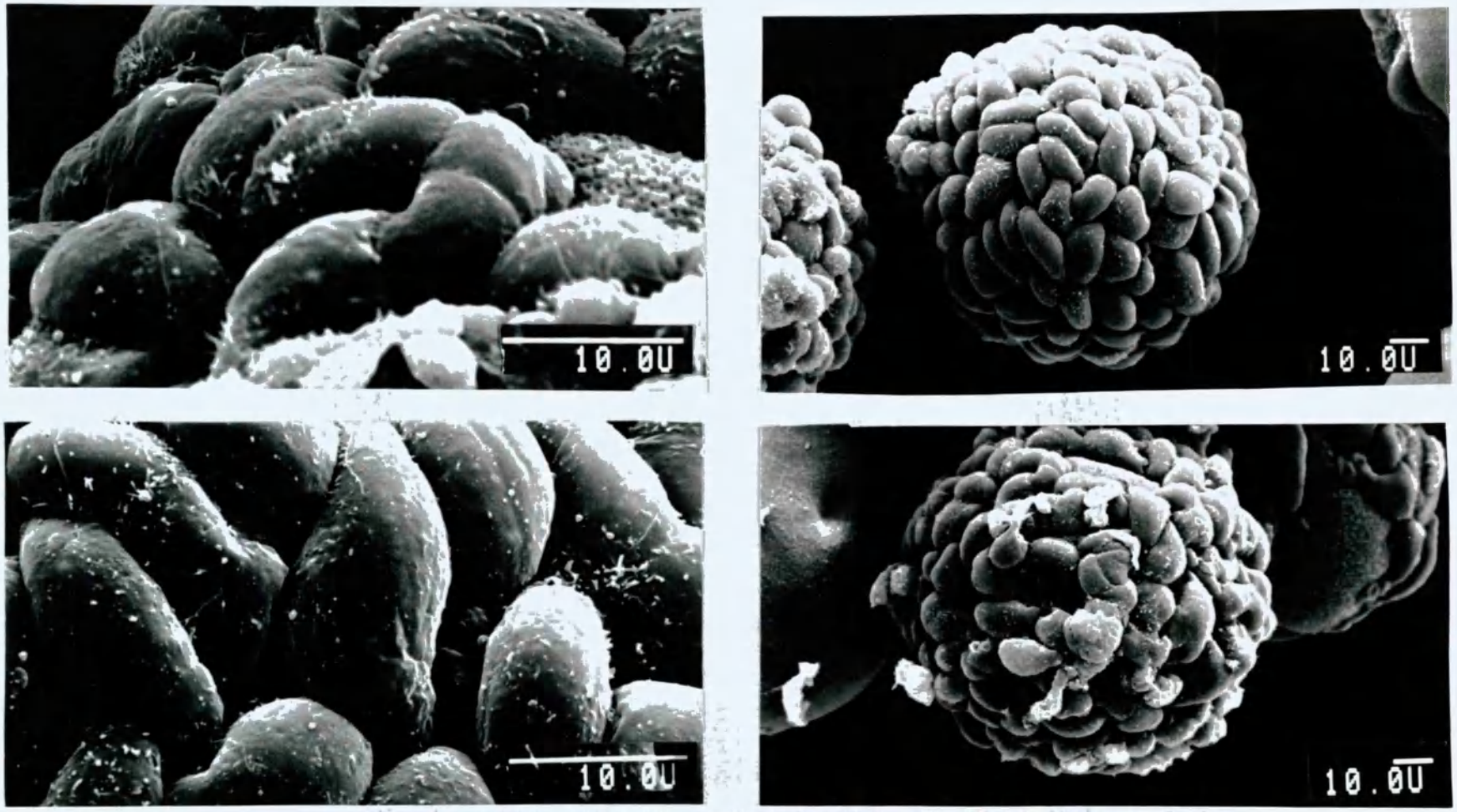


Plate 11(a): S.E.M. of HepG2 cells on Cytodex 2 beads: Single pass perfusion

For 0hrs at magnification X3000 (top left) and 0hrs at magnification X660 (top right)

For 2hrs at magnification X3000 (bottom left) and 2hrs at magnification X660 (bottom right)

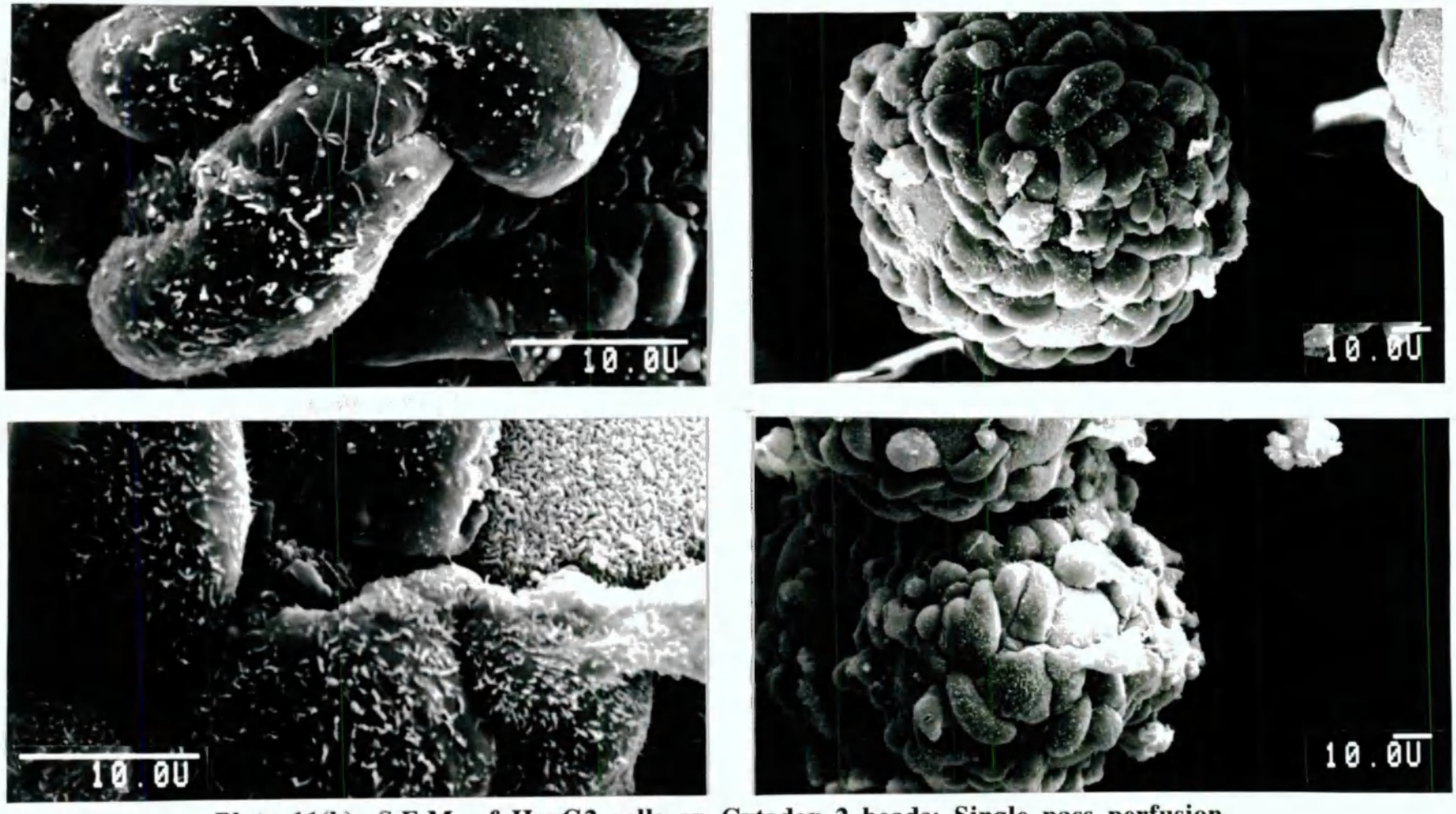


Plate 11(b): S.E.M. of HepG2 cells on Cytodex 2 beads: Single pass perfusion

For 6hrs at magnification X3000 (top left) and 6hrs at magnification X660 (top right)

For 24hrs at magnification X3000 (bottom left) and 24hrs at magnification X660 (bottom right)

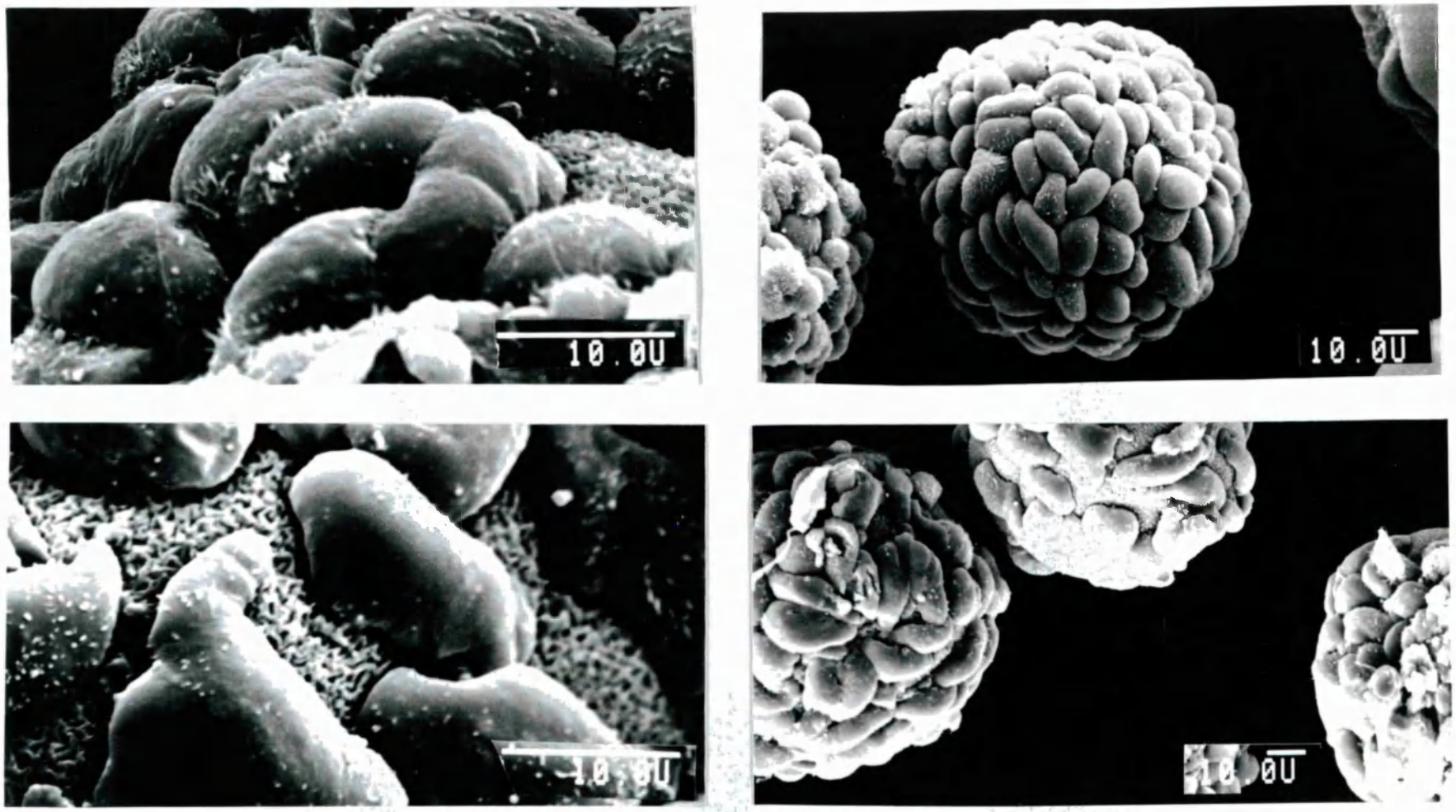


Plate 12(a): S.E.M. of HepG2 cells on Cytodex 2 beads: Reperfusion

For 0hrs at magnification X3000 (top left) and 0hrs at magnification X660 (top right)

For 2hrs at magnification X3000 (bottom left) and 2hrs at magnification X660 (bottom right)

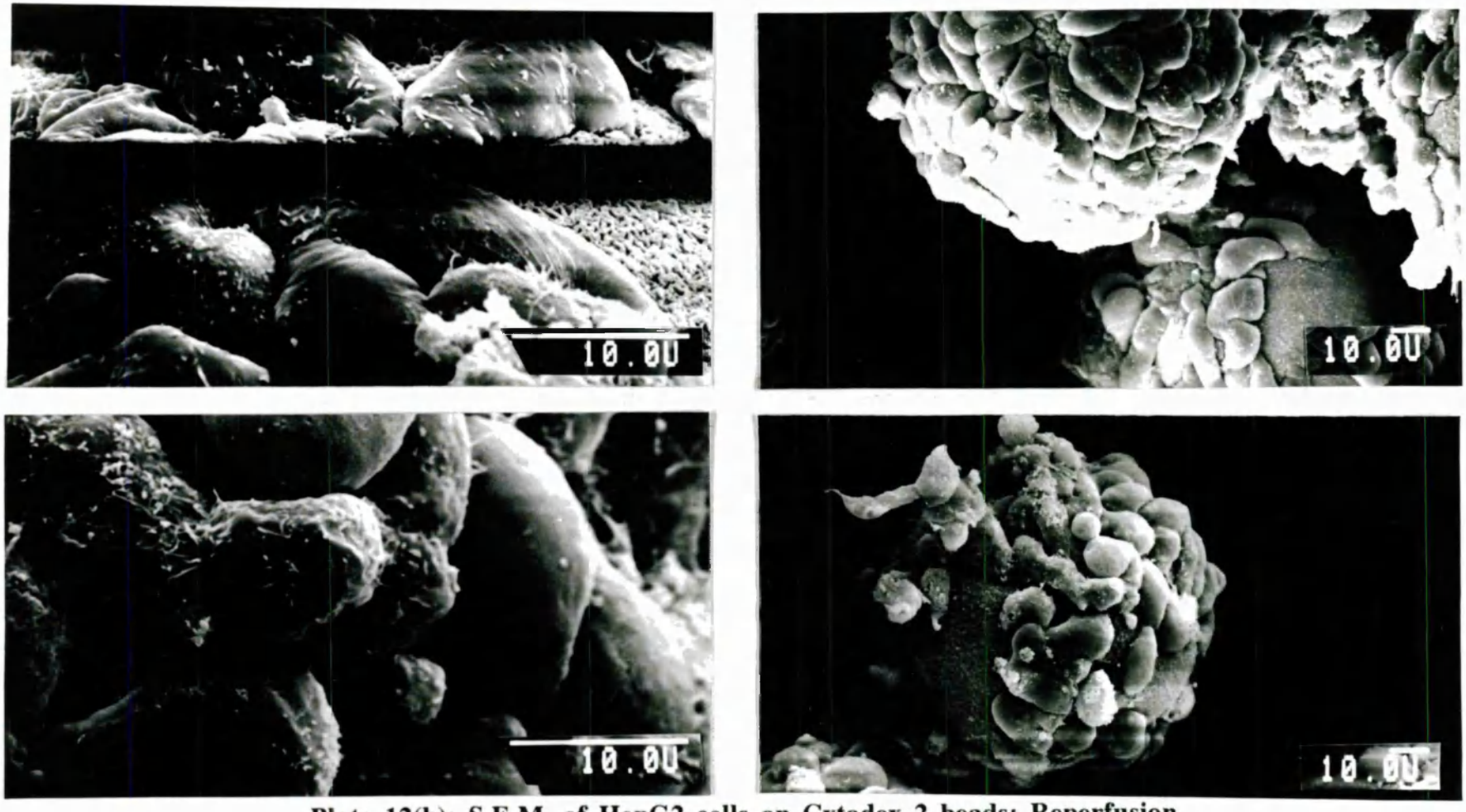


Plate 12(b): S.E.M. of HepG2 cells on Cytodex 2 beads: Reperfusion

For 6hrs at magnification X3000 (top left) and 6hrs at magnification X660 (top right)

For 24 hrs at magnification X3000 (bottom left) and 24hrs at magnification X660 (bottom right)

0.05M sodium cacodylate in 2% gluteraldehyde (osmolality for fixation discussed in Chapter 7.). The results can be seen in S.E.M. photographs plates 11(a) and 11(b) for single pass perfusion, and plates 12(a) and 12(b) for reperfusion. These S.E.M.'s demonstrate that increasing perfusion time produced increased numbers of microvilli, and that the number of microvilli per cell appeared to be greater in cells which had been fed by single pass perfusion, than in cells which had been reperfused.

The presence of higher numbers of microvilli on the cell surface may be an indication of the state of health of the cell. Bylock A. et al [Bylock A. et al., 1979] also found that microvilli were present in greater numbers on the surface of endothelial cells from the uterine arteries of women non-smokers, as compared to women smokers. This information implies that cells fed by single pass perfusion (receiving continuous fresh 10%BLPDS in RPMI medium) microscopically appear more healthy than those that were fed by reperfused medium (3mls 10%BLPDS in RPMI medium). Apart from the number of microvilli the appearance of these cells, that is, the actual overall shape, size and cell surface seemed to be the same throughout the time course.

LDL receptor activity: Since the results from the protein and scanning electron microscopy of the HepG2 cells, on Cytodex 2 beads indicated normal healthy cells throughout the time window of the perfusion method, the next stage was to measure the LDL receptor activity of cells on beads before perfusion and of cells on beads which had been fed by either single pass perfusion or reperfusion. The results are shown in Figure 6.3. (Receptor mediated binding and uptake at 37°C, on cells which have been grown on Cytodex 2 beads. Single pass versus reperfusion). The results here showed that there was no significant difference in receptor activity between zero time and twenty-two hours reperfusion ($P=0.18$), whereas there was a significant difference in receptor activity following single pass perfusion ($P=0.05$), and no increase in receptor activity following reperfusion.

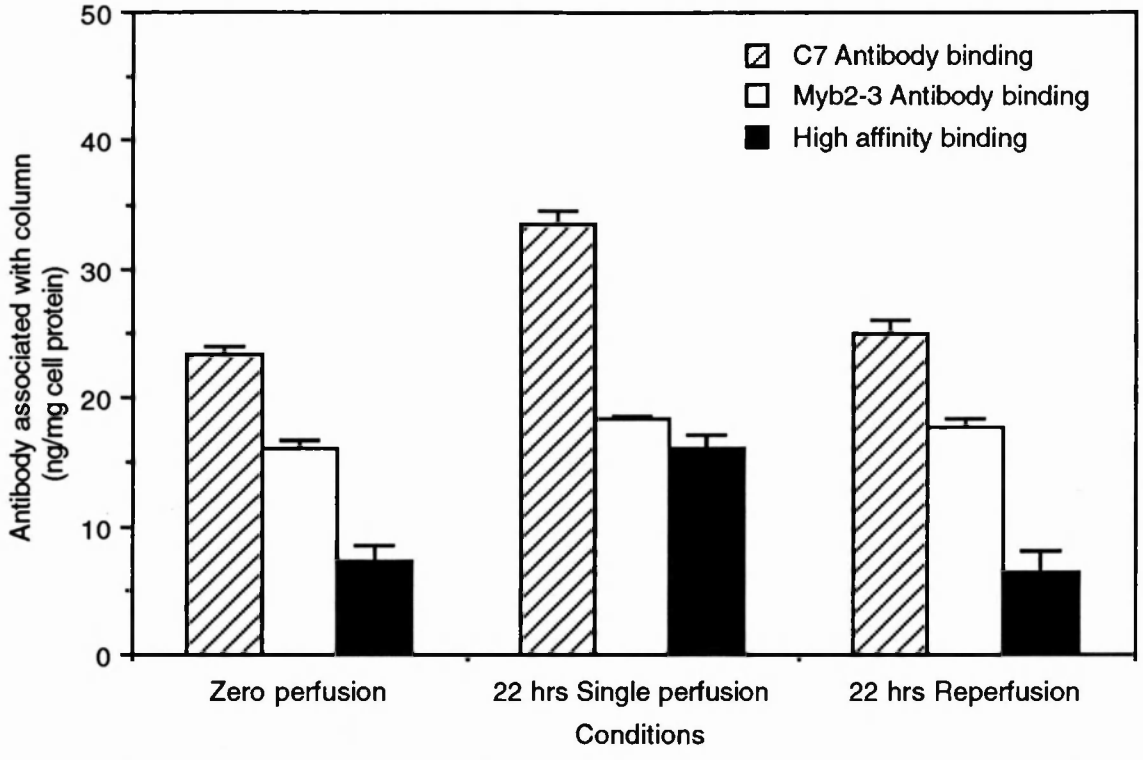


Figure 6.3. Receptor mediated binding and uptake at 37°C, on HepG2 cells which have been grown on Cytodex 2 beads. Single pass perfusion versus reperfusion over a 22 hour period.

Standard method for plating, attachment and pouring of 500µl columns. Reperfusion was performed by using 3 mls. of 10%BLPDS, while single pass perfusion (single perfusion), involved using a continuous fresh supply of 10%BLPDS. Flow rate of both systems was 1ml/hour.

The LDL receptor mediated binding and uptake assay was performed at time zero and twenty-two hours after perfusion. Six columns were used for each condition. Three columns for total binding (^{125}I-C7), and three columns for non-specific binding (^{125}I-Myb$^{2-3}$). High affinity binding was calculated by subtracting non-specific from total binding. Protein assays were performed using the Lowry method.

Plate assays were performed at time point zero, and twenty-two hours to validate the antibodies.

This later observation agrees with the findings demonstrated in Figure 6.1., where there was no change in receptor activity following reperfusion.

The elevated LDL receptor level produced in the single pass perfusion system, compared to the reperfused system could be due to a number of things e.g.

1. The nutrients in the reperfused system being slowly depleted.
2. The waste products from the cells in the reperfused system changing the environment, and thereby decreasing the demand for cholesterol via the LDL receptor.
3. Lipoproteins etc. secreted by HepG2 cells, which in the reperfused system, as in cells in dishes, remained in the environment of the cells, thus decreasing the LDL receptor level by negative feedback.

6.4b. The effect of conditioned medium on the LDL receptor in the column system.

Having established that there was a difference in the LDL receptor activity of HepG2 cells on Cytodex 2 beads in the column system being fed by single pass perfusion compared to reperfusion, it was important to attempt to identify the reason.

The purpose of the next experiment was to determine whether the lower LDL receptor activity observed in the cells which had been reperfused was due to the depletion of the nutrients and/or build up of small molecular weight metabolites; or whether it was due to secretion of factors (e.g. lipoproteins) which acted on the secreting cells. Therefore medium was conditioned by HepG2 cells and dialysed against fresh RPMI to replenish nutrients and to remove waste products.

Conditioned medium was produced when HepG2 cells had been grown to confluence in flasks, then washed and re-fed with 10%BLPDS. After the initial twenty-four hours the medium was removed (not centrifuged) and dialysed against fresh RPMI. Control medium

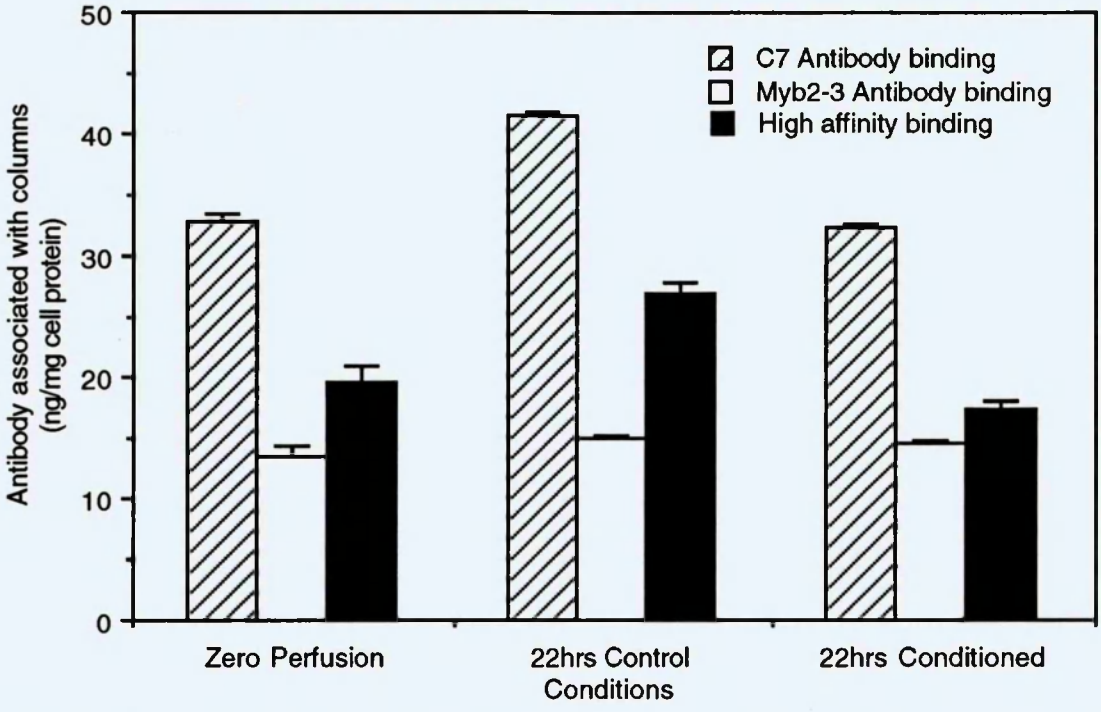


Figure 6.4. Binding and uptake at 37°C, on HepG2 cells which have been grown on Cytodex 2 beads. Single pass of conditioned medium versus control medium.

Standard method for plating, attachment, and pouring of 500µl cell/bead columns.

Conditioned medium was made by incubating 6 x 175cm^2 flasks, confluent with HepG2 cells with 30 mls 10%BLPDS for twenty-four hours. The medium was then dialysed against fresh RPMI, at a ratio of 1:3 overnight at 4°C. Control medium was prepared in the same way as conditioned medium, but in the absence of cells.

Single pass perfusion was performed using both types of prepared medium. Six columns were required for each condition i.e. three columns for total binding (^{125}I-C7), and three columns for non-specific binding (^{125}I-Myb$^{2-3}$). High affinity binding was calculated by subtracting non-specific from total binding. Protein assays were performed using the Lowry method. Data calculated in the same way as in Figure 6.3.

Control: Controls as per Figure 6.3.

was produced by the same method in the absence of cells. Conditioned medium was then used to perfuse cells on beads and the LDL receptor activity was measured. The results are shown in Figure 6.4. (Binding and uptake at 37°C, on HepG2 cells which have been grown on Cytodex 2 beads. Single pass perfusion of conditioned medium versus control medium). This showed that cells which had been fed medium which had been exposed to cells and subsequently dialysed (conditioned medium) caused a significantly lower receptor activity, than that of cells which was fed control medium (not exposed to cells). The P value in this case was 0.02. These results are similar to the results obtained in Figure 6.3. where a significant difference in receptor activity was obtained between single pass perfusion versus reperfusion (P=0.05). This result is also agrees with those observed in Figure 6.1. and Figure 6.3. in that no change in receptor activity could be seen in cells that had been fed by conditioned medium. The medium differed in Figure 6.4. and that of Figure 6.1. and Figure 6.3. in that the medium had been dialysed overnight at 4°C against fresh RPMI (ratio conditioned to fresh medium 1:3) to help replenish nutrient used by the cells. It also diluted any bile salts and waste products produced by the cells (large molecules such as lipoproteins etc. would remain in the dialysed medium, since the molecular weight cut-off of dialysis membrane is 12 kilo Daltons). This therefore indicated that the reduction of LDL receptors on the HepG2 cells which had been fed by dialysed conditioned medium was most probably due to something other than depletion of nutrients, or build up of waste such as bile salts.

HepG2 cells secrete apo B-containing lipoproteins which are known to down regulate LDL receptor expression and it is possible that the presence of these lipoproteins caused the lower receptor activity in perfused cells. To address this possibility HepG2 cells were treated with varying concentrations of glucose, as glucose has been shown to increase apo B secretion [Arrol S. et al., 1991]. The conditioned medium was then used to treat perfused cells prior to receptor assay.

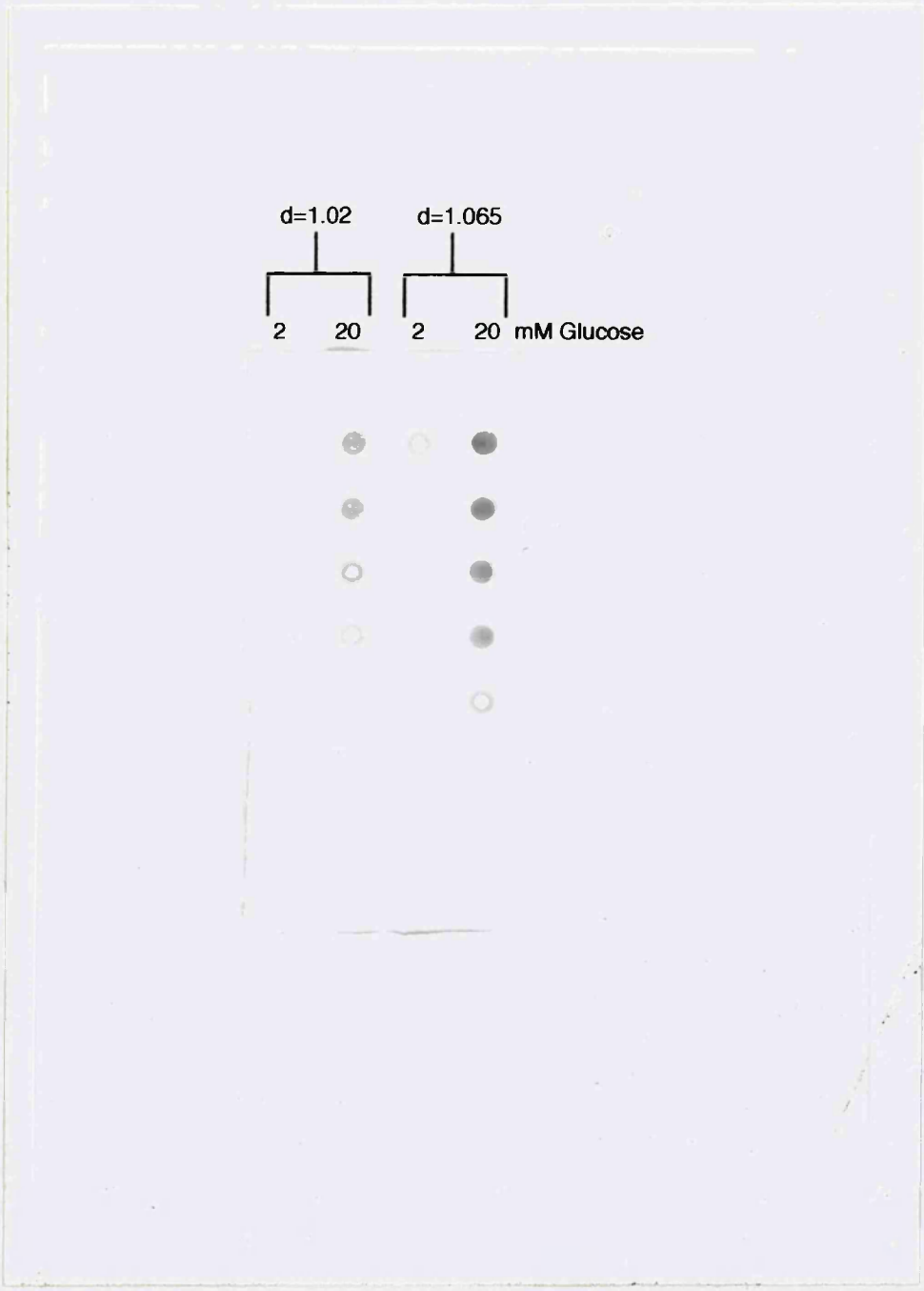


Figure 6.5A. Dot Blot.

HepG2 cells were plated out in 175cm^2 flasks in R10+Glu. and grown to confluence. The flasks were then washed with PBS and re-fed with 35 mls of 10%BLPDS and 2mM or 20mM glucose (six flasks per concentration). Aliquots were then taken and the density changed using 1.020g/ml, or 1.065g/ml KBr before ultra centrifugation at 45000rpm for 16 hours. The top 1cm of solution was then dialysed extensively before samples were taken and double diluted in 96 well plates, enabling that they could be more easily transferred to nitro-cellulose using the Bio Dot (Bio Rad) apparatus (see Chapter 2 for method).

Double dilutions of LDL (1000 - 8ng) were tested on the Dot Blot as a control (results not shown).

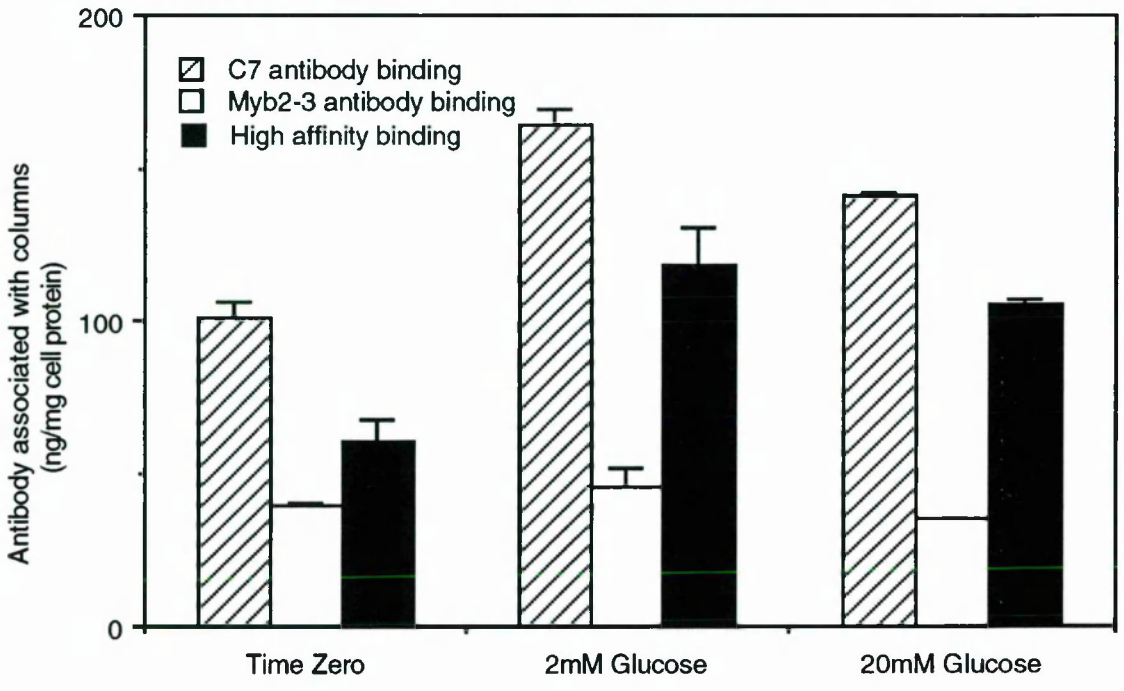


Figure 6.5B. Measurement of antibody binding and uptake at 37°C (ng/mg cell protein), on HepG2 cells grown on Cytodex 2 beads, then single pass perfused for twenty-four hours with 10%BLPDS containing 2mM or 20mM glucose.

HepG2 cells were plated out in 175cm^2 flasks in R10+Glu. and grown to confluence. The flasks were then washed with PBS and re-fed with 35 mls of 10% BLPDS and 2mM or 20mM glucose (six flasks per concentration). After twenty-four hours the conditioned medium was removed, and placed in dialyses tubing and dialysed against fresh medium overnight at 4°C. The conditioned medium was then filtered through a 0.22µM filter and single pass perfused in the column system for twenty-four hours (six columns per glucose concentration). At the end of this period the columns were assayed at 37°C, using 3µg/ml C7 to measure total binding, and 3µg/ml Myb$^{2-3}$ to measure non-specific binding. High affinity binding was calculated by subtracting non-specific from total binding.

In this experiment HepG2 cells were grown to confluence in flasks, then washed and re-fed with 10%BLPDS containing 2mM or 20mM glucose (only 2mM and 20mM glucose concentrations were chosen due to the limited number of channels available, and because this fell within the range used by Arrol S et al [Arrol S et al., 1991]). After twenty-four hours aliquots were taken of each glucose medium concentration for measurement of VLDL and LDL by the Dot Blot method (see Chapter 2.). The results can be seen in Figure 6.5A. The remainder of the medium was dialysed overnight against fresh $RPMI_{1640}$ (glucose concentration 10mM) then filtered and single pass perfused for twenty-four hours, in six columns per glucose concentration. The following day the LDL receptor activity of the cells was assayed at 37°C in the normal way using 3µg/ml ^{125}I-C7 (total binding) and 3µg/ml ^{125}I-Myb$^{2-3}$ (non-specific binding), high affinity was calculated by subtracting non-specific from total binding. The results obtained in Figure 6.5A. showed that at all dilutions there was a marked increase in lipoproteins secreted in the d=1.02-1.065 range (LDL range), compared to that of the d=1.006-1.02 range (IDL/VLDL range). This agrees with observation made by Arrol S et al [Arrol S et al., 1991] in that HepG2 cells can synthesised and secrete lipoproteins in the VLDL [Zannis et al ., 1981] and LDL [Rash et al 1981] range, but it appears that the apo B-containing lipoproteins that are secreted have the buoyancy of LDL rather than VLDL. The results obtained shown in Figure 6.5B. (Measurement of the antibody binding and uptake at 37°C ng/mg cell protein, on HepG2 cells grown on Cytodex 2 beads, then single pass perfused for twenty-four hours with 10%BLPDS containing 2mM or 20mM glucose) showed that despite the pronounced difference in apo B secretion caused by the conditioning cells exposed to the 2mM and 20mM glucose, there was no significant difference in the LDL receptor activity in the cells perfused with the two medium, that is P=0.28.

6.4c. To establish whether the LDL receptor activity on HepG2 cells in the perfused system could be up and down regulated.

The last set of experiments were to establish if the LDL receptor could be up and down regulated by drugs, such as Ketoconazole and 25 hydroxycholesterol in this perfusion

system, together with the effect if any of these drugs being fed by single pass perfusion (continuous fresh medium) compared with that of reperfusion (3mls recycled medium) on the LDL receptor.

Rationale for use of 25 hydroxycholesterol and Ketoconazole to regulate the LDL receptor activity.

LDL receptors are controlled to a large extent by a sterol mediated negative feedback mechanism exerted at the level of transcription of the LDL receptor gene [Südhof et al., 1987: Dawson et al., 1988: Osborne et al., 1988]. Hydroxycholesterols such as 25 hydroxycholesterol act as potent inhibitors of the expression of LDL receptor and of HMG CoA reductase. The concentration of 25 hydroxycholesterol used in the present experiments was 2.5μM. This constantly caused a reduction of approximately 50% in LDL receptor activity in the HepG2 cells when grown in the presence of 10%BLPDS on tissue culture plastic (results not shown). Brown et al 1975 [Brown et al., 1975] showed that 1.5μM 25 hydroxycholesterol gave approximately a 60% reduction in binding activity in human fibroblasts. Kandutsch and Chen in 1974 [Kandutsch and Chen 1974] using cultured mouse cells found that 3μm 25 hydroxycholesterol gave a 50% reduction in receptor binding activity. Using 2.5μM 25 hydroxycholesterol to down-regulate the LDL receptor activity gave these experiments the resolving power needed. That is, if any differences could be observed between single pass and reperfusion on the LDL receptor binding activity (ng/mg cell protein), then further increase or decrease of the LDL receptor binding would be observed.

Ketoconazole was the drug chosen to up-regulate the LDL receptor. This is an anti-fungal drug, which was found to inhibit several enzymes involved in cholesterol synthesis and metabolism [Feldman 1986]. This drug is known to disrupt cholesterol homeostasis at a number of points due to its properties as a cytochrome P450 inhibitor.

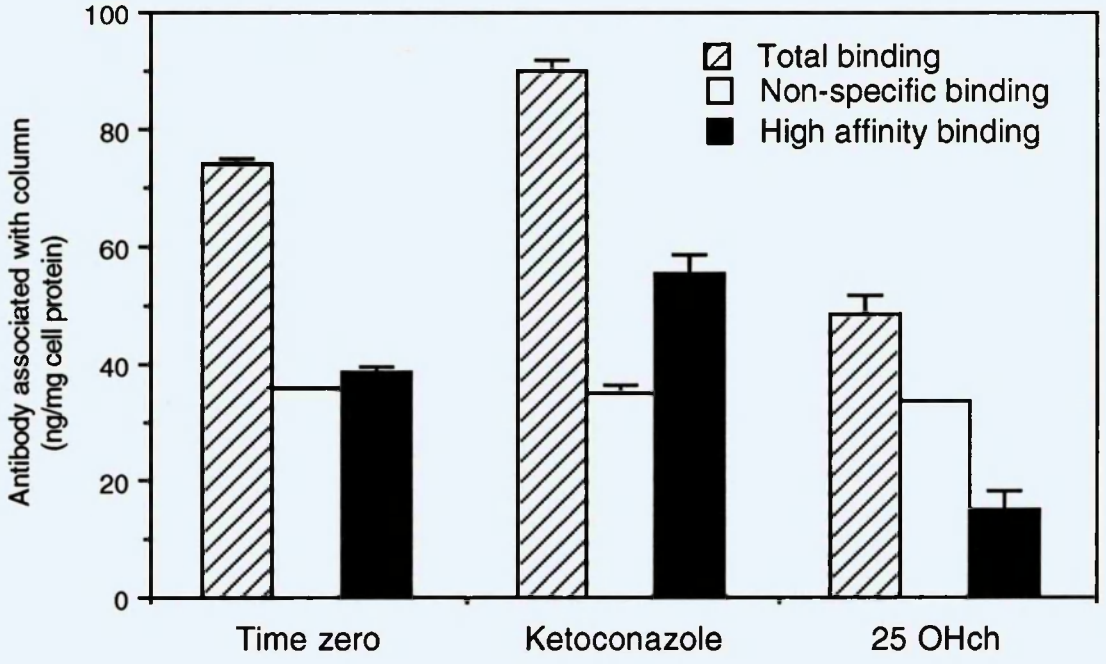


Figure 6.6A. Single pass perfusion of 10%BLPDS in the presence of 30μM Ketoconazole versus 10%BLPDS in the presence of 2.5μM 25 hydroxycholesterol.

Standard method for plating, attachment and pouring of 500μl columns was used. Cells were fed by single pass perfusion for twenty-two hours in medium containing 10% BLPDS in the presence of 30μM Ketoconazole or 2.5μM 25 hydroxycholesterol (25 OHch).

For the measurement of binding and uptake six columns were used for each condition i.e. time zero, twenty-two hours incubated with ketoconazole, and twenty-two hours incubated with 25 OHch. Three columns for total binding (3μg/ml ^{125}I-C7) and three for non-specific binding (3μg/ml ^{125}I-Myb$^{2-3}$). High affinity binding was calculated by subtracting non-specific from total binding. Protein assays were performed using the Lowry method.

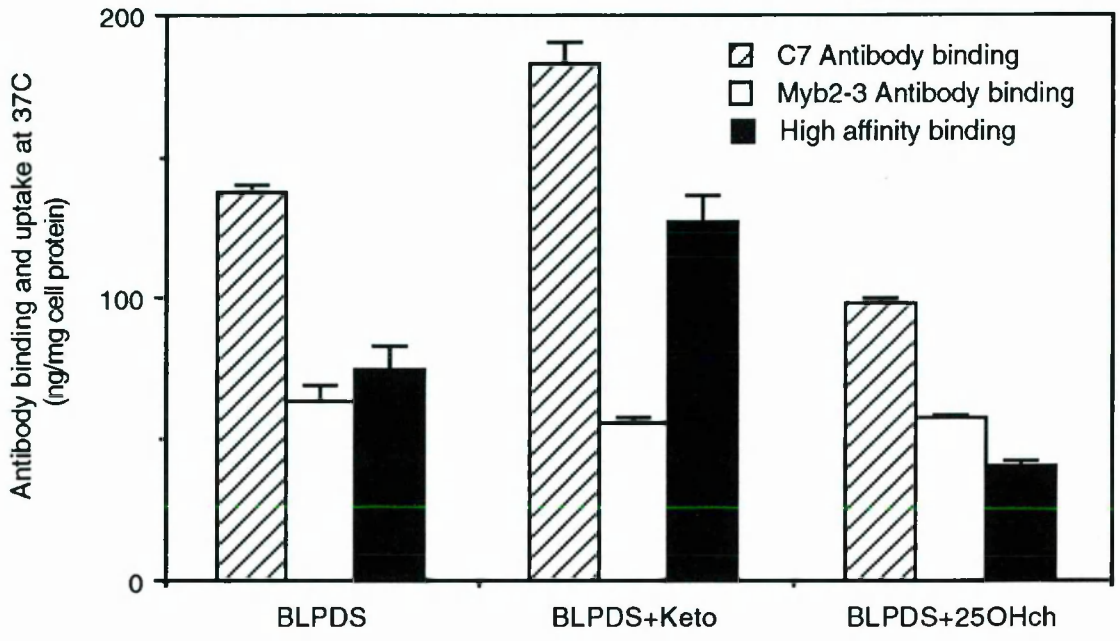


Figure 6.6B. Plate assay performed at the same time as that of twenty-two hours perfusion with 30μM Ketoconazole or 2.5μM 25 hydroxycholesterol in the column system.

HepG2 cells were plated out from the same batch of cells, at the same time as those subcultured for inoculation of the Cytodex beads. These cells were plated out onto Falcon 24 well multiwell plates at a density of 0.2 x 10^6 HepG2 per well. Six wells were plated out for each condition i.e. six wells for 10%BLPDS, six wells for 10%BLPDS + 30μM Ketoconazole, and six wells for 2.5μM 25 hydroxycholesterol. On day two the cells were washed with PBS and fed with appropriate test medium (as used in the perfusion system). On day three the cells were assayed at the same time as those in the twenty-two hour single pass perfusion system, using 3μg/ml ^{125}I-C7 to measure total binding, 3μg/ml ^{125}I-Myb$^{2-3}$ to measure non-specific binding. High affinity binding was calculated by subtracting non-specific from total binding. Protein assays were performed using the Lowry method.

At concentrations of 5-50μM, Ketoconazole inhibits cholesterol synthesis by inhibiting one of the enzymes in the bio-synthesis pathway (lanosterol 14 α demethylase). It is believed that cholesterol must be converted to an oxysterol in order to down-regulate LDL receptor expression. At very high concentrations (50-100μM), Ketoconazole is thought to block all oxysterol formation and therefore at high concentrations negative feed back is eliminated and LDL receptor expression is very high [Gibbons 1983].

The concentration of Ketoconazole used in these experiments was 30μM. This gave an increase of 80-100% up-regulation of receptor binding activity in the HepG2 cells grown on tissue culture plastic, in the presence of 10%BLPDS (results not shown). Gupta et al 1986 [Gupta et al., 1986] also found similar results of 100% increase when treatment of HepG2 cells with 30μM Ketoconazole was used. Higher concentrations of Ketoconazole were not used in any of these experiments due to the general appearance of the cells which indicated that the level of drug concentration was cytotoxic (that is cells coming off the dishes while being assayed; very low cell proteins (data not shown)).

The first experiment in this final set of experiments was carried out to find if the LDL receptor binding activity could be up- or down-regulated by means of the drugs i.e. Ketoconazole (up-regulator) and 25 hydroxycholesterol (down-regulator) in this perfusion system. This was achieved by pouring 500μl HepG2 cells on beads into columns (previously cultured in 10%BLPDS) as in the standard method. This was then single pass perfused with 10%BLPDS in the presence of 30μM Ketoconazole or 2.5μM 25 hydroxycholesterol for twenty-two hours. At the end of this time the cells were assayed at 37°C using 3μg/ml ^{125}I-C7 to measure total binding and 3μg/ml ^{125}I-Myb$^{2-3}$ to measure non-specific binding. High affinity binding was calculated by subtracting non-specific from total binding. Figure 6.6A. (Single pass perfusion of 10%BLPDS in the presence 30μM ketoconazole versus 10%BLPDS in the presence of 2.5μM 25 hydroxycholesterol) shows that there is a difference between zero hours perfusion and twenty-two hours single pass perfusion of Ketoconazole (44% increase in LDL receptor activity), and between zero

hours and twenty-two hours single pass perfusion with 25 hydroxycholesterol (40% decrease in LDL receptor activity).

However after further examination of previous results, the effect of the two drugs in this system is not so clear. That is single pass perfusion in itself has shown to produce an increase in LDL receptor activity compared to that of zero time perfusion (see Figure 6.3. were single pass perfusion caused an increase of 45% LDL receptor activity). This therefore implied that the effect observed in this experiment could be due to the effect of single pass perfusion rather than the effect of the drug Ketoconazole. Ketoconazole which was freshly prepared at the time of perfusion, was also tested on HepG2 cells plated out on tissue culture plastic, using the same batch of cells as those grown in the columns. These cells however showed an LDL receptor increase of 70% which indicated that the drug Ketoconazole was active (see Figure 6.6B.).

In contrast, the reduction in LDL receptor activity on HepG2 cells in columns, observed when single pass perfused with 25 hydroxycholesterol, not only showed the mathematical difference from time zero (P=0.027, a 53% decrease in receptor activity), but the lack of anticipated increase in the LDL receptor activity caused by single pass perfusion (see Figure 6.3.).

The next experiment carried out was to observe the effect, if any, of drugs on the LDL receptor of HepG2 cells, fed by medium being single pass perfused compared to that of medium being reperfused. This was achieved in the first instance by pouring 500µl cell plus bead columns, then perfusing them with 10%BLPDS plus 2.5 µM 25 hydroxycholesterol. Six columns were fed by single pass perfusion (continuous fresh medium) and six columns were fed by reperfusion (3mls continually recycled) after twenty-two hours the cells were then assayed at 37°C using 3µg/ml ^{125}I-C7 to measure total binding and 3µg/ml ^{125}I-Myb$^{2-3}$ to measure non-specific binding, high affinity binding was calculated by subtraction non-specific from total binding.

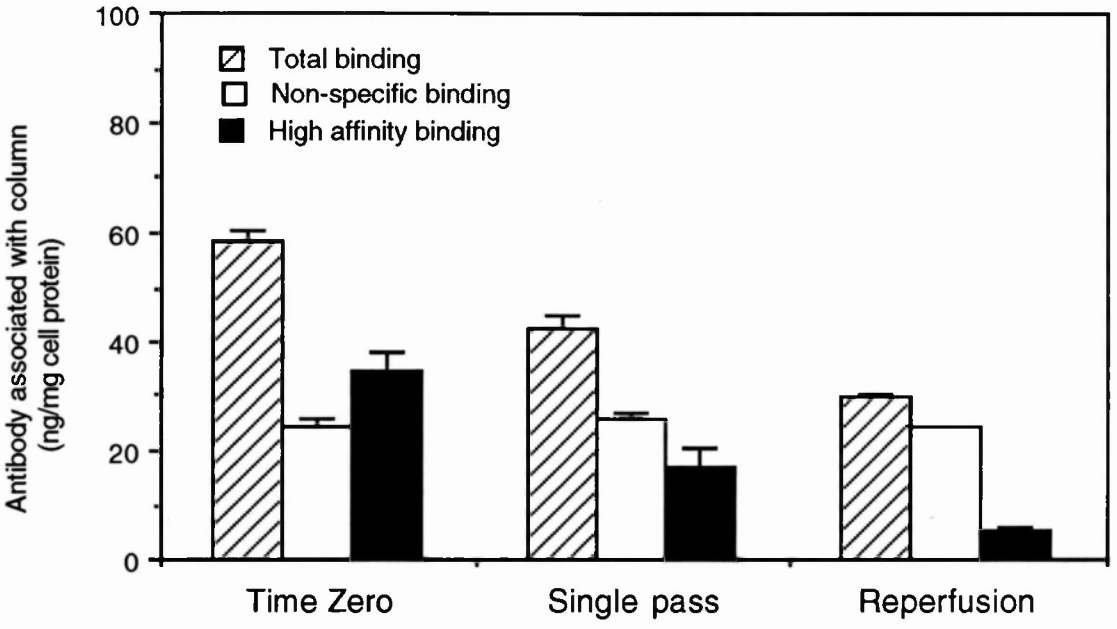


Figure 6.7 25 hydroxycholesterol, single pass verses reperfusion.
Measurement of antibody binding and uptake at 37°C (ng/mg cell protein), on HepG2 cells which have been cultured in 10% BLPDS plus 2.5μM 25 OHch, fed by single pass perfusion or reperfusion.

Standard method for plating, attachment and pouring of 500μl columns. Reperfusion was performed by using 3mls. of 10% BLPDS. While single pass perfusion, involved using a continuous fresh supply of 10% BLPDS.

Six columns were used for each perfusion condition. Three columns for total binding (3μg/ml ^{125}I-C7) and three columns for non-specific binding (3μg/ml ^{125}I-Myb2-3). High affinity was calculated by subtracting non-specific from total binding. Protein assays were performed using the Lowry method.

Figure 6.7. (25 hydroxycholesterol, single pass versus reperfusion) shows the effect of 2.5μM 25 hydroxycholesterol on the LDL receptor of HepG2 cells which had been fed by either single pass perfusion or reperfusion for twenty-two hours. This experiment as in the previous experiment (Figure 6.6.), showed a reduction in the LDL receptor activity from zero time and twenty-two hours single pass perfusion (51% reduction in LDL receptor activity). Plus a further reduction in LDL receptor activity when the cells were fed with 2.5μM 25 hydroxycholesterol by reperfusion (74% reduction in LDL receptor activity).

These experiments are consistent with previous results, in that the LDL receptor activity was greater in cells which had been fed by single pass perfusion as opposed to reperfusion. Similarly the experiment depicted in Figure 6.3. showed a 45% increase in LDL receptor activity when fed by single pass perfusion, and a 10% reduction in receptor activity when fed by reperfusion.

Chapter 7

OBSERVATIONS OF HepG2 CELLS BY ELECTRON MICROSCOPY

7.1. Introduction.

When investigating new and different procedures for growing cells it is important to observe the morphological appearance of those cells. This was achieved, initially, by looking at the HepG2 cells by light microscopy under phase contrast. Then, by fixing the cells with different osmolality concentrations using sodium cacodylate plus gluteraldehyde observations were made by scanning electron microscopy (S.E.M.).

7.2. Method.

7.2.1. Preparation of HepG2 cells grown on collagen coated/non-coated tissue culture plastic, for scanning electron microscopy, using various osmolality concentrations.

So that the effect of the surface growing area and osmolality of the fixative could be observed, HepG2 cells were grown on three types of collagen coated/non coated tissue culture plastic:-

1. Falcon, normal non-coated dishes.
2. Falcon, normal dishes coated with pig collagen type IV(Sigma).
3. Falcon Primaria, (artificial polysine, pig collagen type I, IV and extra cellular matrix).

These were subsequently fixed at three different osmolality concentrations. This was carried out by plating HepG2 cells at a density of 300,000 cells per $10cm^2$ dish, with 4mls of medium R10+Glu (plating see Chapter 2). On day 2 the cells were viewed by light microscopy under phase contrast and sections of cell coated plastic were removed with the aid of a red hot cork borer (preparation of samples in this manner meant that it was impossible to select areas of similar cell density, for comparison of cell

morphology). These sections were then transferred into 0.05M, 0.1M, or 0.2M sodium cacodylate (pH7.4) plus gluteraldehyde and fixed at 4^0C for 2 hours. Once fixation was completed the cells were dehydrated gradually in a graded ethanol series from 30% to 100%. Then transferred to an Akron (Feron) and critical point dried with the use of CO_2 in a Polaron E3000 drying unit, whilst glued with Dag and coated with 30nm of gold using a Polaron E5000 sputter coater. The dried, coated samples were then viewed in a Joel JSM35 scanning electron microscope operated at 15KV.

7.2.2. The Preparation of HepG2 cells grown on Cytodex beads for scanning electron microscopy using various osmolality concentrations.

HepG2 cells were grown on collagen coated (Cytodex 3) and non-collagen coated (Cytodex 2) microcarrier beads and fixed at different osmolality concentrations, enabling the effect of the surface growing area, and osmolality, to be observed on both the cells and beads. This was carried out in the following way:-

HepG2 cells were plated out on Cytodex 2 and Cytodex 3 beads in the normal way (see Chapter 2). On day 2, the cells were washed in large volumes of warm PBS (37^0C), and then examined by phase contrast microscopy. The cells on beads were subsequently transferred to universal tubes and fixed using 0.05M, 0.1M, or 0.2M sodium cacodylate (pH 7.4) plus gluteraldehyde, and prepared for scanning electron microscopy in the same way as for cells grown on tissue culture plastic.

7.3. Observations.

7.3.1. Observations made by scanning electron microscopy of HepG2 cells grown on tissue culture plastic (collagen coated/non-coated), and fixed at various osmolality concentration.

HepG2 cells were grown on three types of Falcon tissue culture plastic, namely; collagen-coated, non-collagen coated and Primaria (see 7.2.1.), and then fixed at three

Plate 13

HepG2 cells grown on non-coated collagen dishes and fixed with 0.05M sodium cacodylate plus glutaraldehyde (pH7.4) Magnification X240

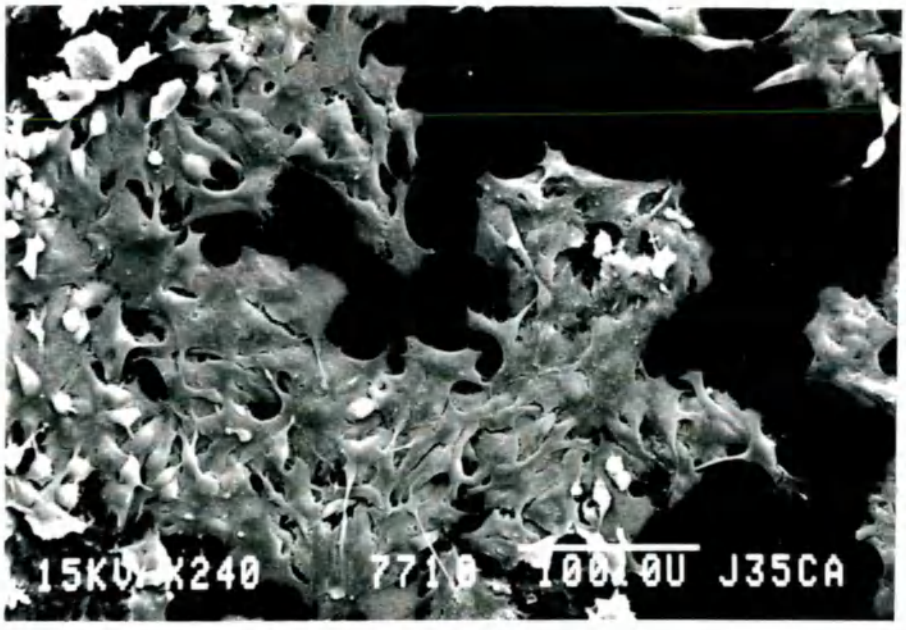


Plate 14

HepG2 cells grown on Primaria dishes and fixed with 0.05M sodium cacodylate plus glutaraldehyde (pH7.4) Magnification X240

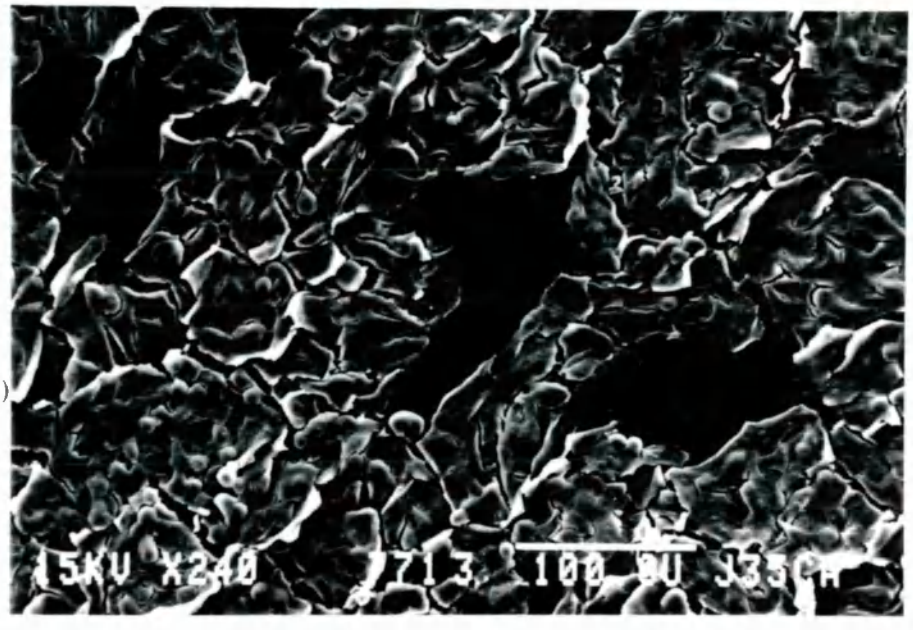


Plate 15

HepG2 cells grown on collagen coated dishes and fixed with 0.05M sodium cacodylate plus glutaraldehyde Magnification X240

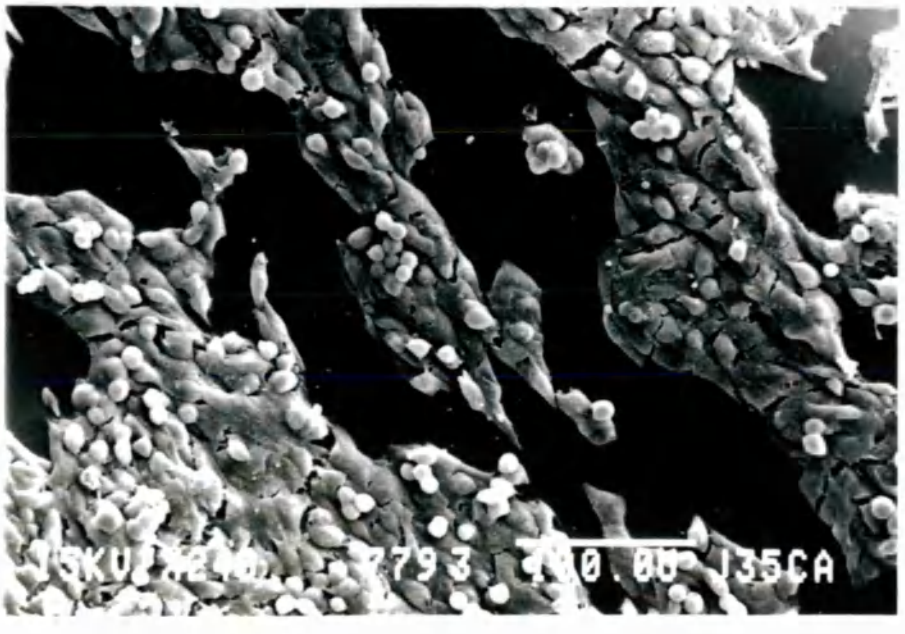


Plate 16

HepG2 cells grown on Primaria dishes and fixed with 0.05M sodium cacodylate plus glutaraldehyde (pH7.4) Magnification X3000

Plate 17

HepG2 cells grown on collagen-coated dishes and fixed with 0.1M sodium cacodylate plus glutaraldehyde (pH7.4) Magnification X3000

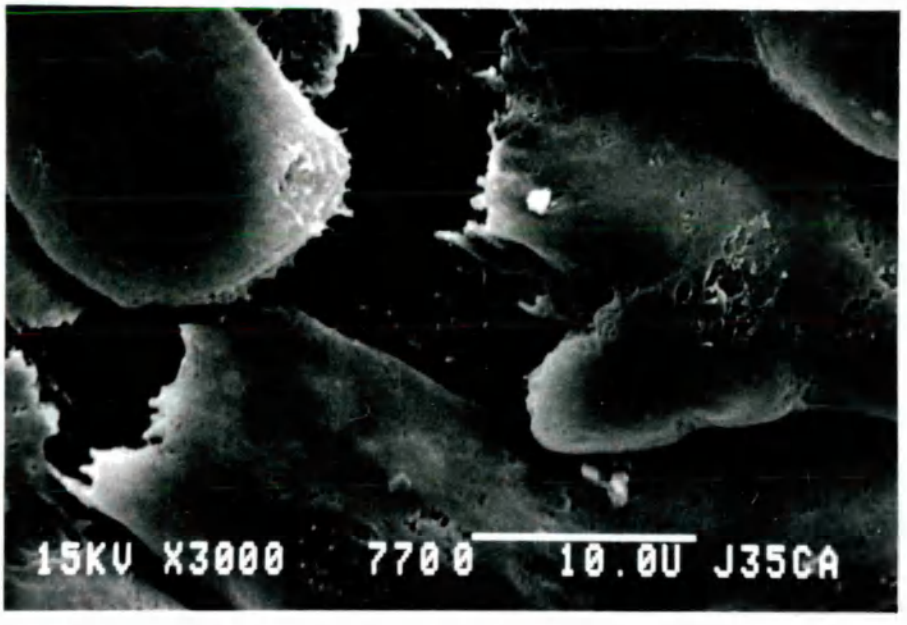


Plate 18

HepG2 cells grown on non-coated collagen dishes and fixed with 0.2M sodium cacodylate plus glutaraldehyde (pH 7.4) Magnification X3000

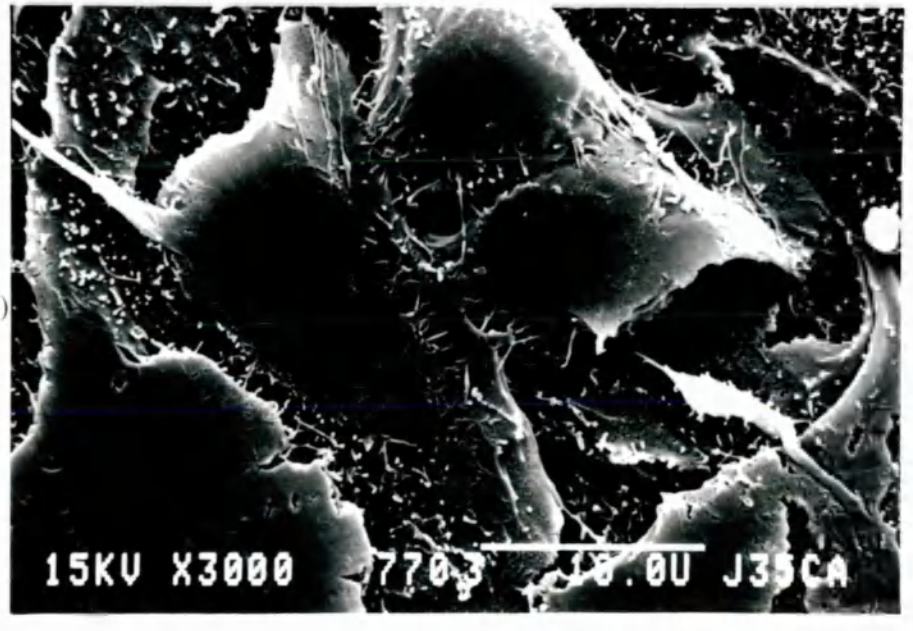


Plate 19

HepG2 cells grown on Cytodex 3 beads and fixed with 0.05M sodium cacodylate plus glutaraldehyde (pH7.4) Magnification X3000

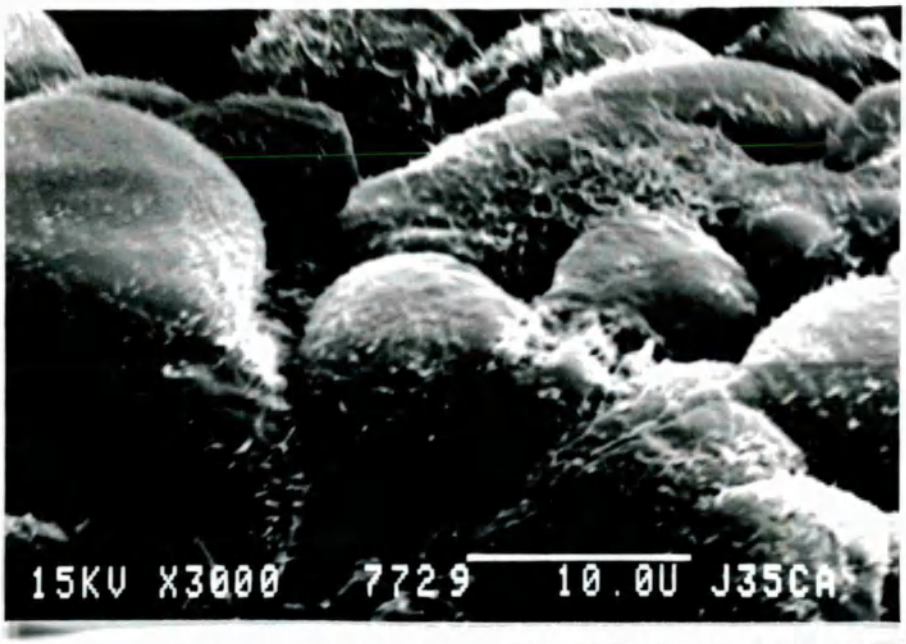


Plate 20

HepG2 cells grown on Cytodex 3 beads and fixed with 0.1M sodium cacodylate plus glutaraldehyde (pH7.4) Magnification X3000

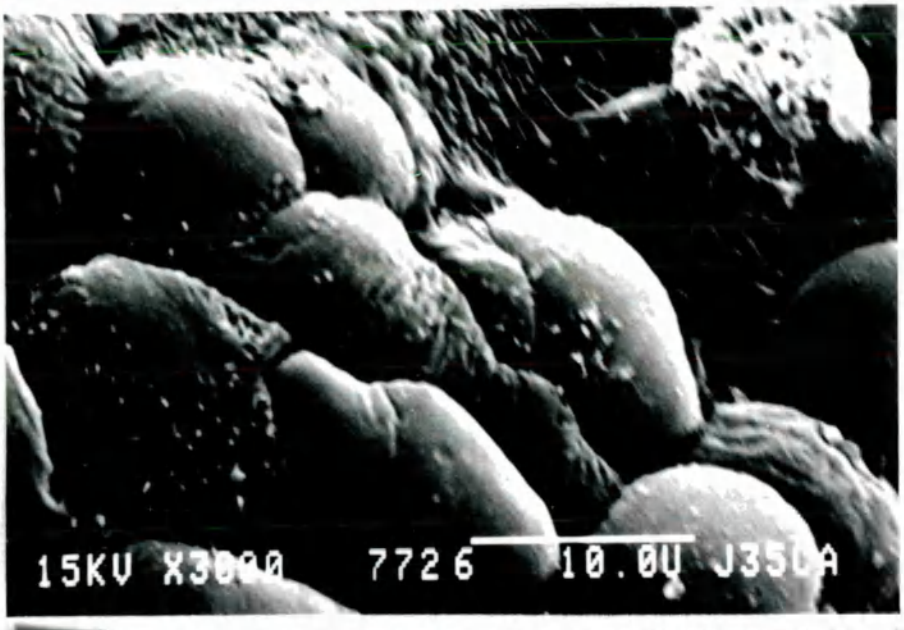
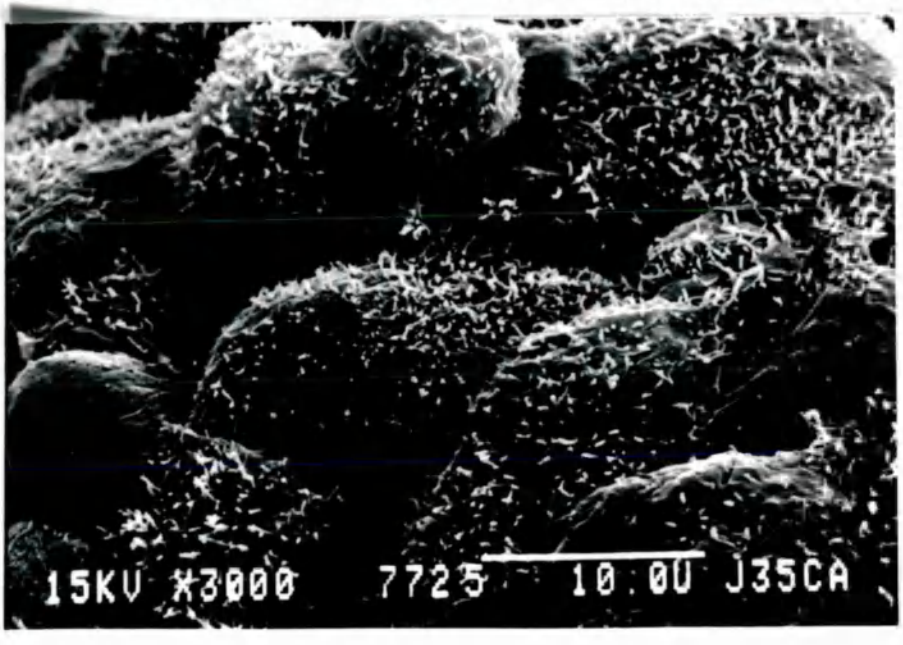


Plate 21

HepG2 cells grown on Cytodex 3 beads and fixed with 0.2M sodium cacodylate plus glutaraldehyde. Magnification X3000



different osmolality concentrations (which was achieved by changing the concentration of sodium cacodylate). Observations were then made by viewing, by, scanning electron microscopy (S.E.M.) and pictures taken at three different magnifications, i.e. 240, 660, and 3000.

Initially, there appeared to be little or no visible morphological difference between the HepG2 cells grown on the three types of coated/non-coated tissue culture plastic, other than perhaps a slight variation in the cell density .

After further examination however, slight variation could be observed in that HepG2 cells grown on non collagen coated tissue culture plastic, appeared more spiky and spread, when compared to HepG2 cells grown on the two types of collagen coated plastic. (Plate 13,14,15.)

The effect of different osmolality conditions showed the same morphological characteristics on HepG2 cells grown on all three types of collagen coated/non coated tissue culture plastic i.e.:-

0.05M sodium cacodylate; which is hypotonic to the cells, shows the cells to have swollen and pulled away, from each other, and in the example of cells grown on Falcon Primaria plastic magnification 3000, the cells can clearly be seen to have burst. The cells also exhibit very small numbers of microvilli. (Plate 16)

0.1M sodium cacodylate; which is isotonic to the cells, shows minimal shrinkage and microvilli. The example chosen to show this was Falcon dishes coated with type IV collagen, magnification 3000. (Plate 17)

0.2M sodium cacodylate; which is hypertonic to the cells, shows the cells have contracted in size and produced large numbers of microvilli. The example chosen to show this was Falcon non coated plastic magnification 3000. (Plate 18)

7.3.2. Observations made by scanning electron microscopy of HepG2 cells grown on Cytodex beads, fixed at various osmolality concentrations.

HepG2 cells grown on Cytodex 2 and 3, fixed at three different osmolality concentrations, by changing the concentration of sodium cacodylate. Observations were then made by viewing by S.E.M. and pictures taken, at three different magnifications i.e. 240, 660, and 3000.

No obvious difference in the morphological appearance of the cells grown on the two types of Cytodex beads was observed.

The different osmolality concentrations showed the same characteristics on both types of Cytodex beads, similar to those observed on HepG2 cells grown on tissue culture plastic, that is:-

0.05M sodium cacodylate; which is hypotonic to the cells shows minimal microvilli (Plate 19 HepG2 cells grown on Cytodex 3 microcarrier beads, magnification X3000).

0.1M sodium cacodylate; which is isotonic to the cells shows minimal microvilli (Plate 20 HepG2 cells grown on Cytodex 3 microcarrier beads, magnification X3000).

0.2M sodium cacodylate; which is hypertonic to the cells shows large numbers of microvilli.

(Plate 21 HepG2 cells grown on Cytodex 3 microcarrier beads, magnification X3000).

The different osmolality concentrations, however, did have a profound effect on the beads themselves, in that:-

0.05M sodium cacodylate (hypotonic) kept the beads swollen and spherical so that cells could be easily viewed without any distortion from the beads.

0.1M sodium cacodylate (isotonic) slightly dehydrated the beads, giving some distortion.

0.2M sodium cacodylate (hypertonic) dehydrated the beads, making them collapse in areas.

7.4. Summary of observations made by scanning electron microscopy.

When growing cells under different conditions to the norm, it is important to observe the morphology for any unusual features that might give an indication to poor health of those cells. It is also important to realise that when one is viewing cells, any alteration in the environment, for example fixing, pressurising, and freezing, will have some effect on the morphological appearance of those cells.

The effects observed in these experiments, showed that when cells were grown on different types of treated tissue culture plastic that cells grown on non-collagen coated dishes exhibited a slightly spiky appearance compared to their collagen coated counterparts.

The osmotic concentration of the cells fixed on beads and on tissue culture plastic, all showed the same characteristics in that:-

0.05M sodium cacodylate (hypotonic) solution produced swollen cells with small numbers of microvilli.

0.1M sodium cacodylate (isotonic) solution showed minimal shrinkage and microvilli.

0.2M sodium cacodylate (hypertonic) solution showed shrinkage and large numbers of microvilli.

Cells grown on Cytodex microcarrier beads were best viewed when fixed under slightly hypotonic conditions (plates 22 and 23), so that the beads themselves did not collapse and distort the cells morphological appearance further, than those caused by their change in environment.

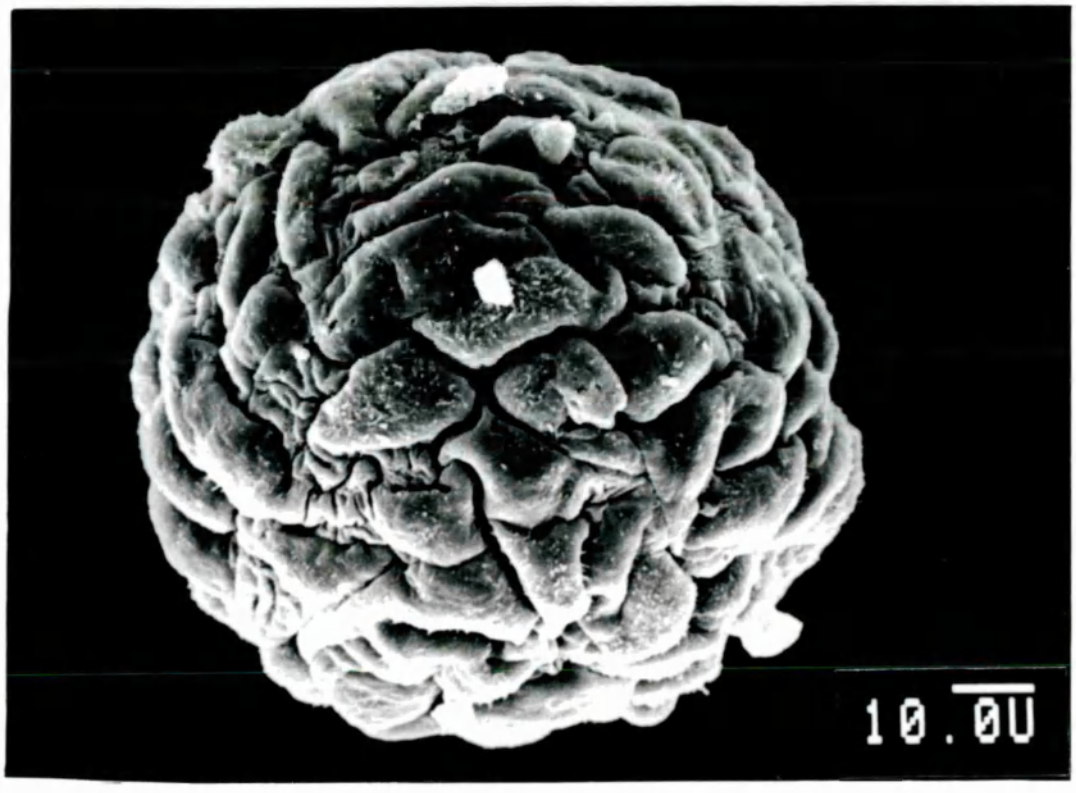


Plate 22 (top) HepG2 cells grown on Cytodex 2 beads and fixed with 0.05M sodium cacodylate plus glutaraldehyde (pH7.4). magnification X240.

Plate 23 (bottom) HepG2 cells grown on Cytodex 2 beads and fixed with 0.05M sodium cacodylate plus glutaraldehyde (pH7.4). Magnification X660.

Chapter 8

DISCUSSION AND CONCLUSION

This thesis describes the design of a method whereby the liver hepatocyte cell line, HepG2, was put into a column system and then fed by perfusion, either with constant fresh medium (single pass), or by medium being continually recycled (reperfusion). The LDL receptor activity of the cells on beads was then measured before and after various perfusion conditions.

The experimental work was conveniently considered in four separate sections.

8.1. Optimising conditions for establishing HepG2 cells on Cytodex beads.

8.1.a. The optimal concentration of HepG2 cells to Cytodex microcarrier beads.

The first concentration tried was that used by Athanase Visvikis [Visvikis et al 1990] of three- thousand cells per cm^2 of microcarrier beads, which was found to be too sparse for our needs. The optimal concentration found to produce a reasonable LDL receptor value compared to that of HepG2 cells grown on tissue culture plastic in the shortest period of time was sixty-thousand HepG2 cells per cm^2 of microcarrier bead.

8.1.b. The type of Cytodex bead most suitable to use.

Pharmacia produce three types of Cytodex beads all of which supported the growth of HepG2 cells. The reason for choosing Cytodex two was that Cytodex 1 which is charged throughout the bead has been reported by Pharmacia themselves to produce in some cases leakage, this may or may not have had some effect on the non-specific binding, of the LDL receptor binding assay performed at the end of perfusion, so therefore could not be used. Cytodex 3 on the other hand was coated with pig collagen type 1, which we had not worked with in the past, therefore making it impossible to make any comparisons with this method and work carried out previously.

8.1.c. Effects of stirring and attachment of HepG2 cells onto microcarrier beads.

This involved several different stages from growing HepG2 cells on Cytodex beads in static bacteriological Petri-dishes, to setting up stirrer bottles at different speeds for the first twenty-four hours and then later twenty-four and forty-eight hours. The optimal speed was found to be that of 20rpm (half final medium volume for the first three hours) for the first twenty-four hours, this was so that cells and beads could collide and attach more easily. Then increased to 40 rpm thereafter enabling the cells on beads to remain in suspension and not clump together too readily.

8.1.d. The period of time to allow the cells on beads to grow.

A high density of HepG2 cells to bead surface area was chosen so that the minimal amount of time (2 days) would be required in this very labour intensive method to produce healthy microscopically looking cells, with which an LDL receptor binding assay could be achieved.

8.1.2. The development of a column system for perfusion, either constant fresh medium (single pass) or by medium being constantly recycled (reperfusion).

This involved designing a pumped system which could be used for perfusion, over varying time intervals in a 37°C, 5% CO_2 incubator. The system was designed to allow both single pass perfusion (an open system which enabled medium to flow gently through the column, so that cells received constant fresh medium) and reperfusion (closed system with medium being continually recycled). The materials of the column plus beads had to be chosen to be suitable for performing an LDL receptor binding and uptake assay. In particular materials had to be selected that minimised non-specific binding of the ligands used to probe the LDL receptor.

Various column sizes and dimensions (1 ml, 2 ml, 5 ml, syringes) were assessed to determine the practicability of which the cells on beads could be easily poured into columns, and that those columns (cells plus beads in the syringe) would be retained

without getting blocked or dislodged. The syringe size chosen from these experiments was 2 ml. Next plugs of various types for the column were tested for their ability to retain the confluent HepG2 cells on beads, and not to bind non-specifically to the ligand used to probe the LDL receptor. This involved a wide range of products from cotton and polyester fabrics, glass fibre wool and commercial filters from Millipore, Sortorus, and Spectra/Mesh. The eventual product chosen was a polypropylene filter from Millipore with a pore size of 25μ. This allowed medium to flow through easily, with minimal non-specific binding occurring in the LDL receptor assay. The flow system was then investigated. Early experiments allowing the medium to flow through the column by gravity alone were found to give varying flow rates, therefore a Watson and Marlow peristaltic pump was employed which maintained the constant flow rate required. The pump speed chosen was 1 ml per hour which gave a gentle flow rate, enabling the HepG2 cells to be bathed in medium with minimal turbulence and disruption in the environment of the cells in culture.

The volume of medium used in the recycled perfusion system was decided upon by comparison of protein values obtained from HepG2 cells grown to confluence in a 1.77cm^2 dish, with those of cells grown on Cytodex 2 beads and placed in columns. The volume of medium in the dish was 0.5mls and gave a protein value of five to six times lower than that obtained from the cells on beads in columns. Therefore, the volume of medium decided upon for the column system was 3.0 mls.

8.1.3. To investigate ligands which were available for the measurement of the LDL receptor, and find the most suitable for use in this system.

The first method examined (for the determination of the LDL receptor) involved the use of 5μg/ml ^{125}I-LDL in the presence or absence of a large excess of unlabelled LDL (250μg/ml) [Goldstein J.L. and Brown M.S. et al 1976]. In this method ^{125}I-LDL bound to the LDL receptor, any non-specific binding which may occur by this method was determined by ^{125}I-LDL competing with excess unlabelled LDL. High affinity or

receptor-mediated binding was calculated by subtracting non-specific from total. However high pseudo specific (high affinity) binding was produced by the naked polypropylene membrane of the columns themselves, without any cells on beads made this method unsuitable for the measurement of the LDL receptor.

The second method involved the use of ^{125}I-LDL to measure total binding and uptake, and ^{125}I-reductively methylated LDL to measure non-specific binding and uptake. Weisgraber [Weisgraber et al 1978] showed that reductive methylation of LDL only modified the lysine groups, which abolished the binding ability activity of the LDL, but kept the overall positive charge the same. Unfortunately this method also showed that ^{125}I-labelled LDL and reductively methylated LDL bound to the column material. The total unsuitability of this method was demonstrated (Chapter 5.) when HepG2 cells which had been fed by 10%BLPDS in the presence of 25 hydroxycholesterol give greater negative values for the LDL associated with the column, than with naked beads themselves.

As it can be seen from the above the natural ligand (LDL) proved unsuitable for the measurement of the LDL receptor on the HepG2 cells in columns due to the complex non-specific binding of the LDL to the column material, beads, and cells on beads.

Finally, success was achieved using the monoclonal antibody C7 directed against the LDL receptor, and a control monoclonal antibody Myb$^{2-3}$, which was directed against an irrelevant antigen. This assay using the monoclonal antibodies was first validated by the measurement of the LDL receptor activity in the HepG2 cells in monolayer culture, using methods carried out by Beisiegel [Beisiegel et al 1981]. Later the LDL receptor activity was measured on cells grown on beads and poured into columns. Cells on beads were incubated in the presence or absence of 25 hydroxycholesterol for twenty-four hours. Various volumes of the beads were then poured into columns and LDL receptor activity was determined. Reproducible measurement of LDL receptor activity could be obtained

in this system and the expected effect of 25 hydroxycholesterol was obtained (see Chapter 5.).

8.1.4. A measurement of the perfusion column system.

In this section I aimed to:-

Determine whether HepG2 cells could be perfused over a twenty-four hour period in the column system and the effect of the conditioned medium on the LDL receptor in the column system.

The first objective required the demonstration that LDL receptor activity could be measured after cells had been fed by reperfusion over various time intervals up to twenty-four hours. It was then important to find out whether perfusion had damaged the cells. This was assessed in two ways, firstly by performing protein assays and secondly by viewing the cells appearance by scanning electron microscopy.

The protein results obtained showed that the differences obtained when cells had been fed by either single pass perfusion or reperfusion was minimal, and that the differences obtained were most probably due to error in pouring and not necessarily due to perfusion. However, the microscopical appearance of the cells by scanning electron microscopy showed that although the actual overall shape, size and cell surface (shrinkage, holes etc.) seemed the same throughout the time course, increasing numbers of microvilli were observed with increased perfusion time, and the numbers of microvilli appeared to be greater in cells that had been single pass perfused rather than fed reperfusion. This implied, at least microscopically, that cells fed by single pass perfusion (constant fresh medium) rather than reperfusion (3mls of medium continually being recycled) appeared slightly more healthy. Bylock [Bylock et al., 1979] also made reference to the fact that microvilli were found to be more prevalent on the surface of endothelial cells from arteries of women non-smokers, compared to women smokers.

In Chapter 6. it can be seen that HepG2 cells which had been fed by reperfusion had a lower LDL receptor activity than those fed by single pass perfusion. In order to investigate whether the reperfused cells had a lower LDL receptor activity due to essential nutrients being depleted, medium was conditioned by incubation with HepG2 cells in cell culture flasks for twenty-four hours and then dialysed against fresh medium. Single pass perfusion was then performed for twenty-four hours against fresh control medium. The results found showed that there was a reduction in LDL receptor activity in the cells fed with conditioned (dialysed) medium indicating that the reduction was most probably due to something other than depletion of nutrients, or build up of waste such as bile salts.

One of the possibilities for a reduction of the LDL receptor activity could have been due to the fact that HepG2 cells secrete apo B containing lipoproteins which are known to down regulate LDL receptor expression, and that the presence of these lipoproteins caused the lower receptor activity in cells which had been fed by reperfusion. Unfortunately, the increase of glucose concentration from 2mM to 20mM (which should have been enough to increase lipoprotein secretion) in the cell conditioned medium showed no significant difference in high affinity binding of the LDL receptor (P=0.28). It is however possible that the level of secretion of apo B-rich lipoproteins was elevated, but not enough to alter the level of LDL receptor activity.

Further experiments were performed to establish whether the LDL receptor activity on HepG2 cells in the perfused system could be regulated by drugs. This last set of experiments demonstrated that HepG2 cells perfused in the column system for twenty-two hours in the presence of 30μM Ketoconazole up-regulated the LDL receptor, whilst 2.5μM 25 hydroxycholesterol down-regulated the LDL receptor. However, this up-regulation, when compared to previous experiments, may have been due to the effects of single pass perfusion, rather than the drug Ketoconazole itself (as shown in Figure 6.5A, Chapter 6.). It should also be noted that LDL receptor activity was further reduced when

the cells had been fed by reperfusion compared to single pass perfusion in the presence of 25 hydroxycholesterol (see Chapter 6.). This indicated once again that substances secreted by the HepG2 cells regulate the LDL receptor expression, and thus validated the use of this type of system for the study of LDL receptor expression on HepG2 cells.

8.2. Summary of what has been observed, and achieved in the research work leading to this thesis.

1. Optimal conditions for culturing HepG2 cells on microcarrier beads have been established.
2. An assay to determine LDL receptor activity on cells on beads has been developed.
3. A column and perfusion system has been designed that allows the measurement of receptor activity in the HepG2 cells on beads under different perfusion conditions.
4. HepG2 cells on microcarrier beads which have been perfused over various time periods up to twenty-four hours, have been observed by scanning electron microscopy, protein assays, and LDL receptor assays.
5. LDL receptor activity has been found to be lower on cells that had been incubated with medium by reperfusion, compared to that on cells incubated by single pass perfusion.
6. The lower activity on cells incubated by reperfusion did not appear to be due to depletion of an essential nutrient. This strongly suggested that substances secreted by the cells regulated LDL receptor activity.
7. LDL receptor activity on HepG2 cells in the perfused column system were shown to be down-regulated by the drug 25 hydroxycholesterol.
8. The LDL receptor activity was reduced further when reperfused with 2.5μM 25 hydroxycholesterol compared with that of single pass perfusion.

8.3. In the Past.

The microcarrier bead perfused system has predominantly been used in previous work for the study of endothelial cells. This enabled cell secretions and chemical interactions to take place in a system which approached an in vivo situation by an in vitro method, in that, in the vascular system the surface area to volume changed dramatically as a unit volume of blood circulates (1000 times larger in the capillaries than that of larger vessels). The microcarrier culture system enabled cultures to be packed into small columns (e.g. 0.3 to 1.0ml) and study for example:-

A. Binding of thrombin and platelet factor 4, for the demonstration of anticoagulant properties of the cell, for studies of the interaction between endothelium and platelets, and for the demonstration of prostacyclin release [Bush et al., 1982].

B. Release of endothelium-derived relaxing factor (EDRF), and prostacyclin (PGI_2) which increased the amount of cyclic GMP in vascular smooth muscle cells exposed to this conditioned filtrate [Kibira et al., 1991].

C. Permeability of human venous endothelial cell monolayers with regards to the effects of flow rate, thrombin, and cytochalasin [Eaton et al., 1992].

Other studies include:-

1. Pituitary cells in the study of the effect of estradiol and progesterone on relative luteinizing hormone and follicle stimulating hormone release induced from superfused anterior pituitary cell cultures by defined LHRH pulse regimens [Kellom et al., 1991].
2. Culturing of epidermal cells on Cytodex 3 beads for the study of epidermal cell biology and biochemistry [Katayama et al., 1987].
3. Johnson Space centre (NASA) has developed a new Rotating-Wall Vessel for the culture of cells of human adenocarcinoma cell lines HT-29, and HT-29KM so that isolation of growth, regulatory, and structural processes within neoplastic and normal tissue can be studied [Goodwin et al., 1992].

Although the above studies show that several cell types have been studied by growing them on Cytodex microcarrier beads and subjecting them to perfusion no work appears to have been carried out on the human hepatocyte cell line HepG2 with regards to the study of the LDL receptor when grown on Cytodex beads or the effects of perfusion on those cells. As mentioned in Chapters 3 and 6., Visvikis [Visvikis et al., 1990] described the growth of HepG2 cells on Cytodex 3 microcarrier beads in stirrer bottles, but not a column system, to analyse and quantify the production of gamma glutamyltransferase. Courney Harrison [Courney Harrison et al., 1991] used a perfusion system for the study of insulin inhibiting PEPCK mRNA levels stimulated by cAMP and dexamethasone using rat hepatocyte cell line H-4-11-E-C3, rather than the human cell line HepG2.

8.4. Future Investigations.

Studies in cell culture have shown that there are a number of factors which influence the secretion of triacyl glycerols (the major constituent of VLDL) and of apo B (the principle protein in VLDL and LDL). These include cholesterol, fatty acids, insulin and glucose. Some of the results from these studies however are contradictory. For example when oleic acid is added to HepG2 cells, secretion of apo B has been shown to either remain the same [Arrol S. et al., 1991], or to increase [Ellsworth J. L. et al., 1986]. Similarly triacyl glycerol (TG) secretion has been shown to be either unaffected [Arrol s et al., 1991] or increased [Dashti M. and Wolfbauer G. 1987]. In addition some authors report that the apo B secreted from HepG2 cells is contained in particles with densities similar to LDL [Arrol S. et al., 1991; Fuki I.V. et al., 1991] whilst others found most of the apo B in particles with densities similar to VLDL [Dashti M. and Wolfbauer G 1987].

One possible explanation for the discrepancies is the presence of hepatic triglyceride lipase (H-TGL) in the HepG2 cultures. In man H-TGL is secreted by liver parenchymal cells. It does not bind to the hepatocytes but rather to the surface of liver endothelial cells where it hydrolyses triglyceride and phospholipid on plasma lipoproteins [Busch S.J. et al., 1991]. Triglycerides secreted by these cells must therefore be prone to hydrolysis by

H-TGL and this casts doubt on the TG secretion values. Further more hydrolysis of the TG in secreted particles would change the composition and therefor the density of the particles.

A further complication when quantifying lipoprotein secretion in static cell culture studies is the removal of secreted particles via lipoprotein receptors. Particles containing apo B and apo E, secreted by HepG2 cells, are removed from the medium by LDL receptors on the same cells [Fuki et al., 1991].

I believe that both of the above problems would be eliminated using the perfusion system, developed in the present work as secreted lipoproteins would be taken away from the cells and cell-mediated lipolysis or re uptake would be minimised. This would, I believe, make it possible to determine whether the amount of lipoprotein secreted is increased, or whether the composition of the particles is changed, when the cells are treated with; a) cholesterol, b) saturated, monounsaturated or polyunsaturated fatty acids, c) cholesterol and fatty acids combination.

Other investigations;

1. This system would be ideal for the testing of unstable drugs, in that fresh drugs could be fed continuously or by pulsation to the cells by perfusion.
2. It would give the opportunity for co-cultures; in that a mixture of two types of cells could be perfused in the same column system or in tandem column e.g. the influence of HepG2 cells on endothelial cells could be studied.

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